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By

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Dedication

I would like to thank my family for their continued support. My wife, Melissa for her continued assistance and patience with my research. My daughter for helping me keep everything in perspective and her smile for making it all worth the effort.

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Abstract

Novel Mechanisms of Antigen processing that enhance BCG vaccine efficacy

Publication No----------

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Mycobacterium tuberculosis, the causative agent of tuberculosis, is the most lethal single infectious agent afflicting man today causing 2 million deaths per year. The World Health Organization recommends a vaccine as the best option to prevent this disease. The current vaccine, BCG, has a variable efficacy and does not protect adults. It is known that BCG vaccine becomes sequestered in special phagosome compartments of macrophages that do not fuse with lysosomes. Since lysosome fusion is necessary for peptide production and T cell priming leading to protective T_H1 immunity, we hypothesized that vaccine efficacy is reduced and occurs perhaps due to non-lysosome dependent mechanisms. We therefore proposed an in depth analysis of phagosome environment, and its proteome to unravel mechanisms of antigen processing and presentation. We initially discovered that three mechanisms of pH regulation including vacuolar proton ATPase, phagocyte oxidase and superoxide dismutase (SOD) secretion from BCG vaccine affect antigen processing within phagosomes. These studies led to the discovery that a mutant of BCG vaccine which lacked SOD was a better vaccine. Subsequently, the proteomic analysis of vaccine phagosomes led to the discovery of novel protease (γ-secretase) enriched on BCG vaccine phagosomes. We then demonstrated that these proteases generated a peptide from the BCG vaccine which was presented through the MHC-II pathway to T cells and induced a T_H1 response. The specificity of antigen production from γ-secretase was confirmed through siRNA knockdown of the components of the protease namely, nicastrin, presenilin and APH, which led to a decrease in antigen presentation. We therefore conclude that, even though BCG phagosomes are sequestered and do not fuse with lysosomes to generate peptide antigens, there are complex and novel *in situ* mechanisms within phagosomes that are capable of generating an immune response. We conclude that T_H1 immunity to BCG vaccine arises mostly due to non-lysosome dependent immune mechanisms of macrophages and dendritic cells.

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List of BCG Strains Used

BCG-Tice: Sub strain of the original BCG-Pasteur vaccine strain. First obtained in 1934 and is also called BCG-Chicago

BCGΔ*SODA*: Mutated strain of BCG-Tice lacking the SOD gene due to *SODA* gene disruption

BCGΔ*SODASecA2*: Mutated strain of BCG-Tice lacking the SOD and SecA genes due to *SODA and SecA* gene disruptions

BCG-Pasteur: Original mutated strain of the BCG vaccine strains created in 1921, first lyophilized in 1961

CHAPTER 1

INTRODUCTION

Tuberculosis

Tuberculosis (Tb) is the leading cause of death in man due to a single infectious agent and is also the number one killer of people co-infected with Human Immunodeficiency Virus (HIV-1)(1-3). The infection can occur in multiple organs but the most common location of infection is in the lungs, resulting in pulmonary tuberculosis(4-6). Tuberculosis symptoms include chronic cough, blood in the sputum, fever and weight loss (6). Tuberculosis is spread when an infected individual with an active form of the disease coughs. Aerosolizing the bacteria and spreading it in the air, and droplets are summarily inhaled by other individuals(6). The majority of people infected are quickly able to contain the infection within granulomas in lungs. However, approximately five percent of patients develop acute tuberculosis(6). In many individuals, the infection remains in a latent state until the individual becomes immune-compromised, and the infection can take advantage of the person's compromised state. New threats continue to emerge with this disease, including the rise of multi-drug resistant (MDR) infections as well as extensively drug resistant (XDR) infections(7-9).

Mycobacterium tuberculosis

Mycobacterium tuberculosis (*M.tuberculosis; Mtb*) is the causative agent for tuberculosis. The genus *Mycobacterium* was first observed in 1896 by Lehmann and Neumann(10). Mycobacterial members are bacillary shaped organisms. Mycobacteria are approximately 3μm long, gram-positive and stain acid fast. There are three major groups of antigens expressed by mycobacteria, cytoplasmic soluble antigens, secreted antigens and cell-wall lipid-bound insoluble antigens(10), mycobacteria contain large amounts of surface and

soluble lipids and proteins. Mycobacteria are responsible for two of the most serious diseases that plague man, tuberculosis and leprosy (Hansen's disease). *Mycobacterium tuberculosis* was first observed and named by Robert Koch in 1882. Currently, approximately one-third of the world's population is latently infected with *M.tuberculosis*, with a new infection occurring about every second.

TREATMENT AND VACCINATION

There are several options for treatment of tuberculosis, which include the use of first line drugs such as rifampin, pyrazinamide and isoniazid(11-13). While these drugs are bactericidal and can treat the infection, they also cause severe liver toxicity in patients. They are less effective in treatment of MDR or XDR strains of infections, leading to the alternate use of secondary drugs. To prevent individuals from contracting tuberculosis, a vaccine *Bacille Calmette Guerin* (BCG) is used, which is derived from *Mycobacterium bovis*. BCG, is a live attenuated vaccine that has been in use since 1921(14-19). It is one of the safest and most distributed vaccines available, with over one billion doses administered to date. There are over thirteen different strains of BCG available for vaccination, although efficacy of the vaccination varies from zero to eighty percent, with a higher efficacy for protection against childhood pulmonary tuberculosis, but lower efficacy against adult onset tuberculosis. This lack of long term immunity is likely due to the fact that BCG undergoes phagosome maturation arrest that prevents the induction of strong, robust and sustained immune response(14-19). The World Health Organization (WHO) has determined that the best and most cost effective way to quell this disease is with a novel more effective vaccine.

DENDRITIC CELLS AND MACROPHAGES

Dendritic cells and macrophages are the major antigen presenting cells of the immune system. They phagocytose invading organisms, break them down and load peptide fragments onto either MHC class II for presentation to $CD4^+$ T cells, or MHC class I for presentation to $CD8^+$ T cells(20). These are important steps in clearing infection since T cells are capable of feed-back activation of macrophages and DCs through cytokines, enhance oxidant responses, kill pathogens, help their degradation and enhance immune responses, through amplifying peptide production (21,22). A simple difference between macrophages and DCs is that the latter are capable of priming naïve T cells, a process whereby a T cell that has never contacted an antigen will become 'sensitized' and acquire the ability to respond to a repeat dose of antigen, when exposed to the same antigen (23-26). Such T cells can also mature into memory T cells when infection is controlled, and then undergo recall expansion upon re-infection. Macrophages are generally not capable of priming naïve T cells although they can induce expansion of pre-sensitized T cells. Thus, DCs are required for the initial priming phase of infection and macrophages for maintenance of T cell memory. However, this line is blurred since DCs can continuously sample fresh antigens during infection and expand the 'repertoire' of T cells. Thus, effective vaccination efforts lead to expansion of the activity of both DCs and macrophages.

GENERATION OF AN IMMUNE RESPONSE

As soon as DCs and macrophages (antigen presenting cells; APCs) phagocytose either Mtb or BCG vaccine, they are enclosed in a lumen called as the phagosome, which is an invagination of the plasma membrane enclosing the bacterium. The mycobacteria are then

usually degraded by the acidification, proteolysis and peptides are produced to be presented on the surface of the APCs. The change that occurs in phagosome over the course of infection is called 'maturation' that generally results in fusion with lysosomes. The peptides are then recognized by T cell receptor bearing CD4 and CD8 T cells. This generates an immune response. These events are summarized below.

PHAGOSOME MATURATION

The immune response to bacterial invasion involves phagosome maturation, and fusion with the lysosome(27). Typically, a bacterium is endocytosed through receptor mediated uptake, and then resides within an endosomal, membrane bound, compartment, also termed a phagosome. The phagosome then matures by a process of protein turnover, which eventually creates a hostile environment within the phagosome, with a low pH and high proteolytic activity, especially when fused to the lysosome, an organelle containing digestive enzymes(28). The low pH is usually due to vATPase. After fusion with the lysosome, the bacteria are killed by one of the several mechanisms including, but not limited to, phagocyte oxidase, nitric oxide or proteolytic enzymes. Degraded peptides are exported to multi-vesicular bodies called MIIC compartments where they are assembled into the major histocompatibility complex (MHC) class II for transport to the cell surface and presentation to T cells (27). There is a biochemical basis for the ability of Mtb and BCG to cause phagosome maturation arrest and these events are illustrated in figure-2.

Initially, the phagosome is enclosed in rab5 positive early phagosomes. Rab5 is a Ras associated protein, which is a Gtpase. The phagosome then acquires additional proteins

mainly through the generation of PtdIns3P (PI3-phosphate) through the enzymatic action of phosphatidylinositol 3-kinase hVPS34.

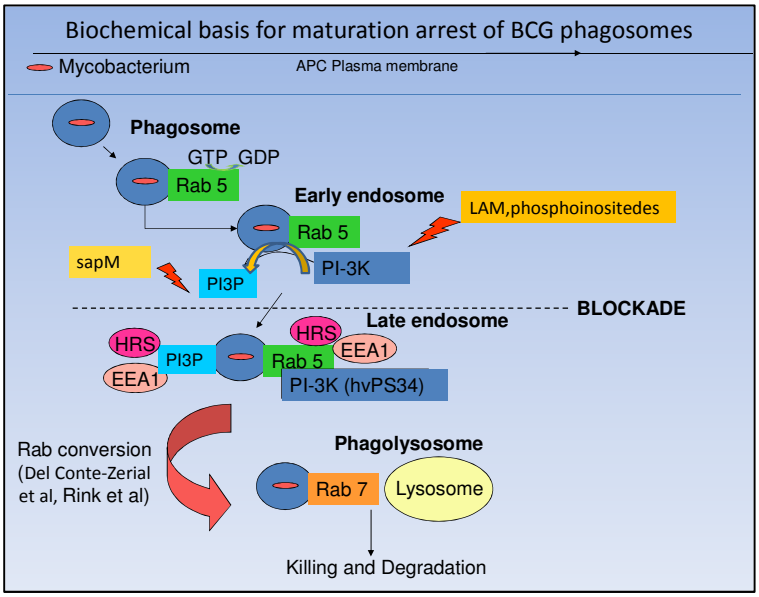


Figure-1: M. tuberculosis and BCG vaccine persist in infected macrophages by blocking Rab conversion and affecting Rab effectors. Type III phosphatidylinositol 3-kinase hVPS34- lipid kinase hVPS34 and its enzymatic product PtdIns3P are critical for the default pathway of phagosomal maturation into phagolysosomes. Mycobacteria block PtdIns3P production and thus arrest phagosomal maturation (29,30).

THE VACUOLAR PROTON ATPASE

The major mechanism that acidifies the phagosomal environment is the vATPase(31). Acidification is important for the activation of some enzymes that are required for the killing and breakdown of Mtb. The other pumps that can influence lumen pH are Na^+/H^+ dependent proton pump and chloride channel (32,33). vATPase is a multi-subunit protein that assembles on the phagosomal membrane and pumps Hydrogen (H^+) ions into the phagosomal lumen, thereby acidifying the lumen (Figure 2). Acidification can be

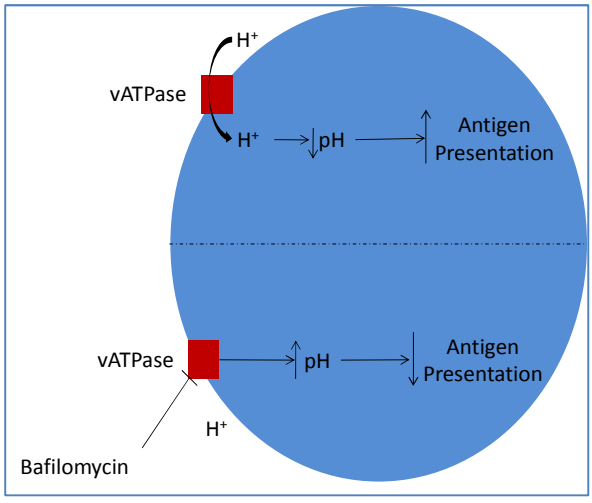


Figure 2: Effect of vacuolar proton ATPase (vATPase) on the regulation of pH inside phagosomes and the effect of bafilomycin on vATPase

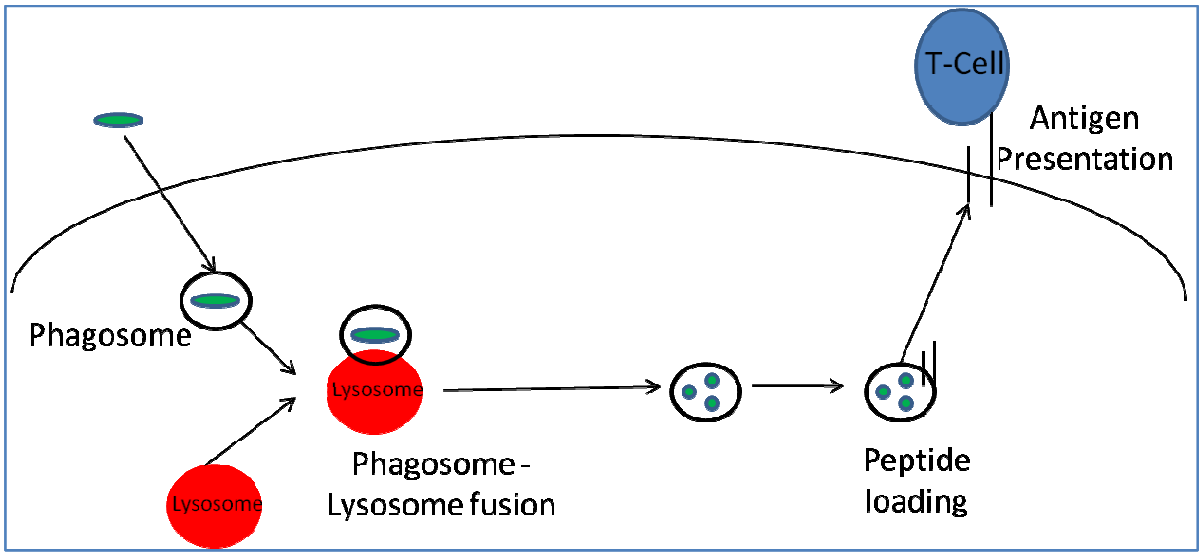
prevented by the addition of Bafilomycin, which specifically inhibits the action of vATPase by preventing the assembly of the V_0 and V_1 components on the phagosomal membrane(34,35). Bafilomycin therefore prevents acidification of phagosome lumen and we demonstrated in an earlier report that it neutralizes acidification of the phagosomes containing Mtb H37Ra within macrophages (36).

TB MATURATION ARREST

One major difficulty with controlling *M.tuberculosis* infection and enhancing vaccine efficacy through the use of BCG is that both strains are able to stall the maturation and fusion with lysosome (37-39). Both BCG and Mtb secrete a phosphatase *sapM* that dephosphorylates the PI3-phosphate on the phagosome membrane (40-42). In addition, both produce several metabolic analogs that compete with host enzymes during the initial events of PI3-phosphate synthesis. Thus, phagosome maturation inhibition allows the virulent Mtb to survive and persist within human hosts. On the other hand, BCG vaccination is unable to induce a protective immune response against Tb because it is not processed by lysosomes (43-46) (47). In the absence of lysosomal fusion, the mycobacterial phagosomes have been found to cleave and produce peptides *in situ*, which is a novel emerging mechanism (48). This is the focus of our proposal.

ANTIGEN PRESENTATION

Once an invading pathogen is degraded in the lysosomes, antigens are loaded onto MHC-II molecules and shuttled to the cell surface to be presented to T cells. T cells, in turn secrete cytokines IFNγ, IL-2 and others that act upon macrophages and DCs to enhance their function. This feedback activation of T_H1 immune response is essential for the control of intracellular infections with Mtb and BCG vaccine (49-53). Evidence in animal models and human infection strongly indicate that it is the $CD4^+$ IFN-γ^+ T cell dependent T_H1



ıccine is taken up
·ine and peptides
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response that is critical for protection against tuberculosis, although $CD8^+$ T cells also play a role particularly against long term infection (54,55). Thus, humans depleted of $CD4^+$ T cells through HIV-1 infection, rapidly succumb to tuberculosis. It is possible that in humans, $CD8^+$ T cells maintain immune surveillance and amplify both $CD8^+$ and $CD8^+$ T cells when infection recurs.

T_H1 IMMUNE RESPONSE

The immune system can respond to infection in multiple ways. An innate immune response provides a short lived immune response(56). However, for long lasting protection, a cell mediated, adaptive immune response is necessary(57). This response can provide protection against specific bacteria and infection and can be classified into two types, T_H1 and T_H2(57). A T_H1 response activates antigen presenting cells such as macrophages and dendritic cells, and also stimulates B cells to produce opsonizing antibodies, while T_H2 responses lead to mostly immunoglobulin responses. It is believed that a strong T_H1 response is needed to contain and eliminate *Mycobacterium tuberculosis*(49-53,58). Tuberculosis infection is characterized by a reduced T_H1 response, but sometimes correlates with increased T_H2 response in humans living in tropical regions. Induction of a strong T_H1 response controlled by T cells is essential for providing protection against infection by Mtb, and this protection is initially induced by Mtb infected dendritic cells. When vaccination is used to induce immunity, DCs are involved. Dendritic cells, while capable of priming T cells may also get, infected with mycobacteria and show decreased maturation and activation (59-61). Such an inhibition helps *M. tuberculosis* to survive within dendritic cells reducing immune responses, similar to the down-regulation of immune responses within macrophages. Thus, both Mtb and BCG persist in host macrophages and DCs for years in a latent state.

STATEMENT OF HYPOTHESIS

The BCG vaccine phagosome is arrested at an immature phagosome stage, although it contains multiple protein components required for antigen processing assembly (48,62-64).

Thus, phagosomes appear to be peptide processing organelles (65). However, our previous observations showed that the pH of the phagosome is near neutral, suggesting weak proteolysis (36). We therefore hypothesize that the efficacy of antigen processing in phagosomes may be dependent on pH regulating factors, although pH-independent, membrane associated processes may also be present. Thus, our aims are as follows.

Aim I: Characterize the BCG phagosome for pH regulating processes that determine efficacy of antigen production.

- *Sub Aim IA*: Characterize proton pumps that acidify the phagosome lumen
- *Sub Aim IB*: Investigate if the phagocyte oxidase that regulates oxidants within phagosomes affects pH of the phagosome and thereby modulates antigen production
- *Sub Aim IC*: Determine if phagosome pH can be modulated through the use of genetic mutants of BCG vaccine

Aim II: Utilize proteomics to identify novel mechanisms that may mediate antigen processing of the BCG vaccine

- *Sub Aim IIA*: Characterize the proteomics of BCG vaccine phagosomes
- *Sub Aim IIB*: Investigate the proteomes of BCG phagosomes from macrophages & DCs
- *Sub Aim IIC*: Investigate function of novel target proteins associated with BCG phagosomes

CHAPTER 2

Characterize the BCG phagosome for pH regulating processes that determine the efficacy of antigen production

CHAPTER 2-1

Characterize the BCG phagosome for pH regulating processes that determine the efficacy of antigen production

Sub Aim IA: Characterize effects of proton pumps that acidify the phagosome lumen

INTRODUCTION

Tuberculosis is the leading cause of death in mankind due to a single infectious agent. The causative organism, *Mycobacterium tuberculosis* (*M.tuberculosis*) is able to parasitize macrophages of man and animals and grow within them. Paradoxically, macrophages are also the cells that are capable of killing these organisms through acidification of bacterial phagosomes, oxidative processes and lysosomal degradation (66-69). *M.tuberculosis* has clearly evolved various mechanisms to neutralize these antimicrobial mechanisms. First, mycobacteria exclude vacuolar proton ATPase (*vATPase*) a major mechanism of phagosome acidification(36). Second, mycobacteria elaborate scavenging lipids, catalase-peroxidase and superoxide dismutases (*SOD*) to neutralize oxidants (70,71). Third, their phagosomes fail to fuse with lysosomes (72-74). The BCG vaccine used to prevent tuberculosis produces antigens largely similar to *M.tuberculosis,* having been derived from *M.bovis,* the pathogen that causes bovine tuberculosis. Interestingly, it seems to have inherited many immune evasion features of *M.tuberculosis* that enable it to sequester in immature phagosomes of macrophages.

We investigated how immunogenic peptides are produced in macrophages and DCs. It was known that, mycobacterial phagosomes could assemble MHC-II complexes that contained an epitope of immuno-dominant antigen-85B (Ag85B) which was then presented to the T cell hybridoma (BB7 T cells), resulting in IL-2 secretion (48). This *in vitro* model facilitated investigating mycobacterial antigen processing in APCs but, the molecular mechanisms remained unclear. We then demonstrated that mouse macrophages infected with BCG vaccine presented Ag85B epitope to T cells, but the efficacy of presentation was reduced compared to macrophages infected with *Mycobacterium tuberculosis* H37Ra, an attenuated

mutant of Mtb H37Rv wild type (36). Additional studies described the key finding that a protease, Cathepsin-D, was required to cleave Ag85B and produce an epitope which in turn, was presented to T cells through the MHC-II pathway (36). These events are summarized in figure-4 below. However, despite progress in our understanding of antigen processing at the phagosome level, significant questions have remained. What are the factors that determine the function of proteases in the phagosome? How does the BCG phagosome maintain its lumen at a near neutral pH? Is it possible to modulate the pH of the phagosomes so that antigen production can be enhanced? To address these questions, we developed two major hypotheses.

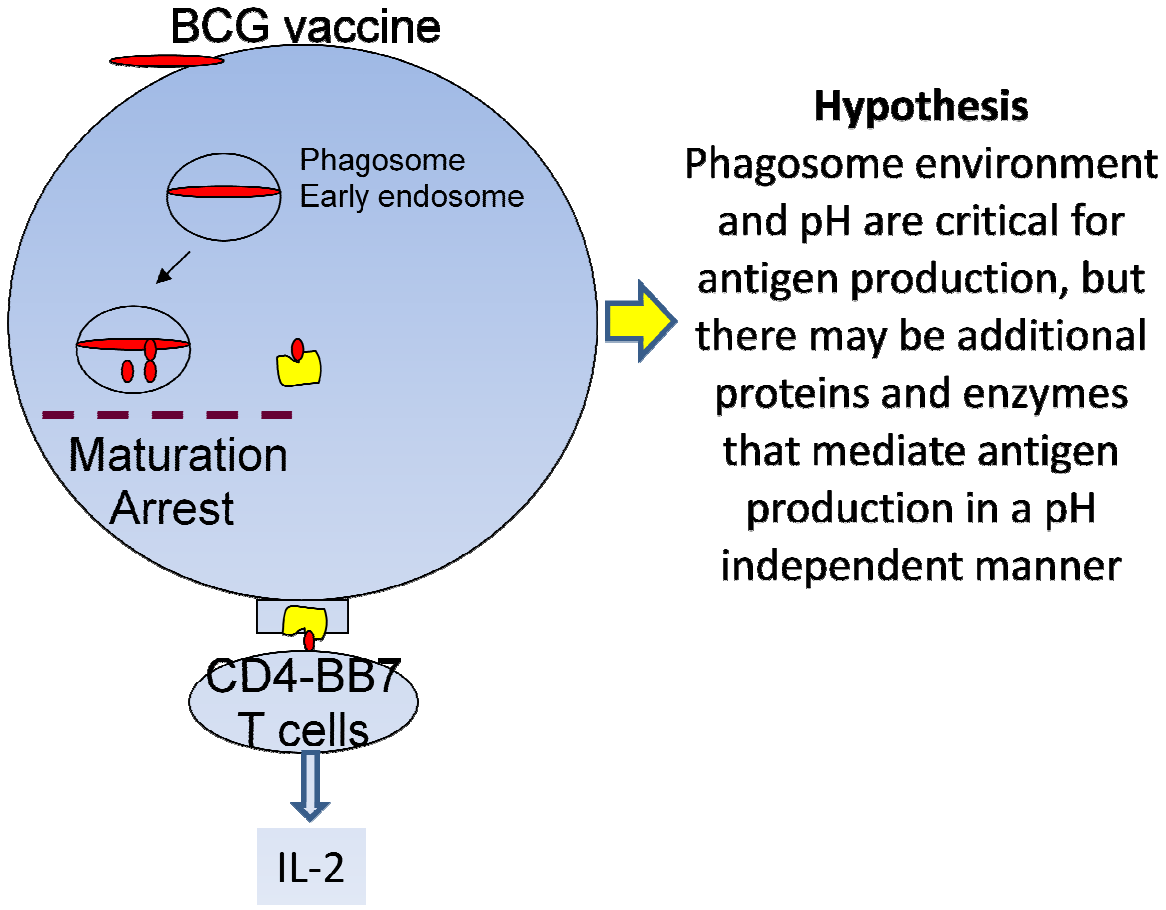


Figure-4: Hypothetical model of the importance of pH during antigen presentation

OVERALL HYPOTHESIS

From published literature it is known that a) the enzyme vATPase pumps protons into phagosomes to make it acidic(36), b) the NADPH oxidase (phagocyte oxidase or phox pump) secretes superoxide (O_2*) that can potentially neutralize incoming protons, thereby increasing the pH (75), and c) BCG vaccine secretes a powerful enzyme, superoxide dismutase (SOD) that dissociates the superoxide into molecular oxygen and water, in a process that increases the pH(76). Thus, the intra-phagosomal pH appears to be the result of at least three converging and regulating factors. Since BCG derived Ag85B can be cleaved by Cathepsin-D only at an acidic pH, we proposed the initial hypothesis that peptide production in phagosomes is dependent upon pH regulation. In this chapter, we specifically asked whether BCG phagosomes have a functional vacuolar proton ATPase pump and whether it influences antigen processing.

METHODS

C57BL/6 mouse derived macrophages and dendritic cells were purified as per previously published methods (METHODS 5-1). They were incubated with varying doses of the vATPase inhibitor, bafilomycin for 4 or 24 hrs and infected with BCG vaccine strain for 4 hours post bafilomycin treatment. Macrophage or DC monolayers were washed and over laid with BB7 T cells, which release IL-2 when Ag85B epitope is presented from macrophages or DCs to T cells (METHODS 5-2). pH of the phagosome was rendered acidic using the calcium ionophore, nigericin. *gfp*BCG phagosomes are green due to neutral pH and were made acidic with nigericin. The effect of vATPase on the pH of the *gfp*BCG phagosomes was determined using bafilomycin blockade and then labeling the macrophages

with lysosensor-yellow-blue dextran (LSY) dye, revealing acidic phagosomes as blue and greenish yellow as near neutral pH phagosomes (METHODS 5-10). The BCG strain used for these experiments was BCG Tice (a sub strain of BCG Pasteur) and kindly supplied by our collaborator, Dr. D. Kernodle from Vanderbilt University. The growth and maintenance of BCG and cell cultures are described under Methods section.

RESULTS

Figure-5 demonstrates that treatment of macrophages and DCs with bafilomycin prior to BCG infection leads to a marked dose dependent inhibition of antigen presentation. Multiple experiments with both macrophages and DCs confirmed the observation (**Table I**). IN contrast global reduction of pH of macrophages using nigericin led to a marked increase in antigen presentation (Figure-6).

In a previous study, we found that acidic pH enhanced the auto-catalytic degradation of 56 kDa immature Cathepsin-D into 30 kDa active enzymatic form (36). Furthermore, BCG phagosomes (pH 6.5) had reduced levels of active Cat-D while Mtb H37Ra phagosomes that acidified (pH 5), had increased levels of Cat-D. We also demonstrated by using siRNA knock-down that Cat-D was directly involved in cleaving Ag85B to produce an epitope that was presented to BB7 T cells.

In order to further determine if bafilomycin induced increase of pH affected the generation of active forms of Cathepsin-D, BCG phagosomes were purified on sucrose gradients and analyzed using western blot for active and inactive forms of Cat-D. Macrophages were activated with IFNγ as a positive control and in addition, phagosomes containing latex beads were purified from naïve macrophages as a negative control. Figure 7 demonstrates that

purified *gfp*BCG phagosomes from bafilomycin treated macrophages show reduced levels of active 30 kDa Cat-D compared to phagosomes from untreated or IFNγ activated macrophages. Latex beads that mature quickly into lysosomes showed active Cat-D even without macrophage activation. Finally, the pH of the phagosomes was estimated using the fluorescent dye LSY that co-localizes with *gfp*BCG phagosomes yielding a blue color when the phagosome lumen pH is acidic. Figure 8A and B illustrate that bafilomycin treatment of macrophages, increases the pH of *gfp*BCG phagosomes so that the proportion of green BCG phagosomes was higher. These data together implicate the vATPase as one major mechanism of acidification of BCG phagosomes in macrophages.

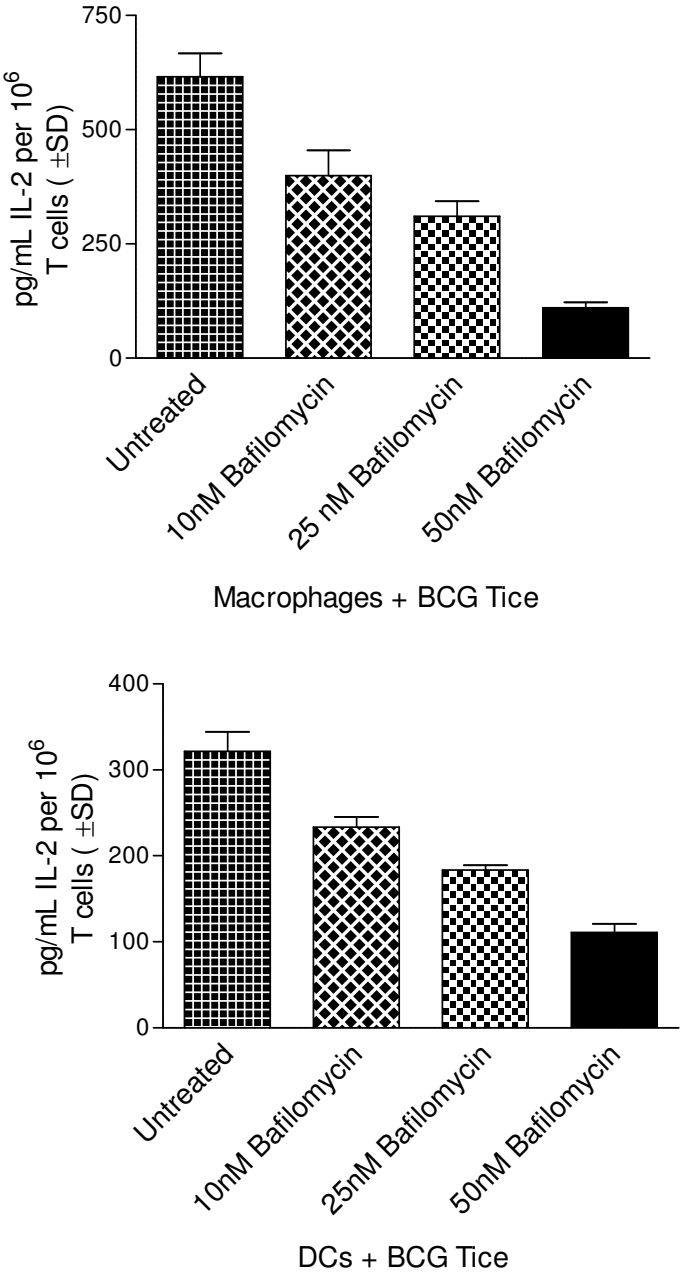


Figure-5: Bafilomycin, an inhibitor of vacuolar proton ATPase (vATPase) leads to reduced antigen 85B presentation in macrophages and dendritic cells*. Primary murine macrophage or dendritic cells were plated into 24 well plates, treated with varying doses of bafilomycin for 24 hours, infected with BCG Tice for 4 hours, washed and then overlaid with BB7 T cells. IL-2 secreted by T cells into the supernatant was measured using sandwich ELISA (data from one of 3 similar experiments).*

Table I: Bafilomycin blockade of vacuolar proton ATPase in two types of antigen presenting cells inhibits Ag85B presentation in vitro*. Macrophages and DCs derived from C57BL/6 mice were tested for the ability to present Ag85B to BB7 T cells and the inhibitory effects of bafilomycin were measured using levels of IL-2 as a parameter.*

Cell Type	Bafilomycin	pg/ml IL-2	SD	n
Primary Dendritic Cell	0	206.60	47.68	2
	13 nM	38.84	0.00	2
	26 nM	43.89	5.28	2
	52 nM	63.64	46.25	2
Primary Dendritic Cell	0	78.35	14.90	2
	13 nM	39.06	2.17	2
	26 nM	51.13	11.79	2
	52 nM	58.81	10.86	2
Primary Dendritic Cell	0	170.00	9.00	2
	13 nM	52.01	6.21	2
	26 nM	64.30	7.45	2
	52 nM	71.33	5.59	2
Primary Macrophage Cell	0	6107.84	2166.96	2
	13 nM	5728.86	3007.87	2
	26 nM	3886.22	2799.95	2
	52 nM	3173.99	748.50	2
Primary Dendritic Cell	0	2605.51	1233.64	2
	13 nM	1005.21	227.92	2
	26 nM	802.35	14.34	2
	52 nM	658.09	186.48	2
Primary Dendritic Cell	0	2605.51	1233.64	2
	13 nM	1016.48	247.04	2
	26 nM	1281.32	168.94	2
	52 nM	1671.27	379.33	2
Primary Macrophage Cell	0	7631.95	2372.56	2
	13 nM	6506.43	924.08	2
	26 nM	4480.83	314.19	2
	52 nM	2056.64	78.55	2
Primary Dendritic Cell	0	2004.37	0.00	2
	13 nM	1042.40	76.50	2
	26 nM	1417.69	14.34	2
	52 nM	1605.90	86.07	2
Primary Macrophage Cell	0	873.1553	60.657	2
	30 nM	880.9536	138.59	2
Primary Macrophage Cell	0	115.3724	9.2659	2
	30 nM	112.2388	18.532	2

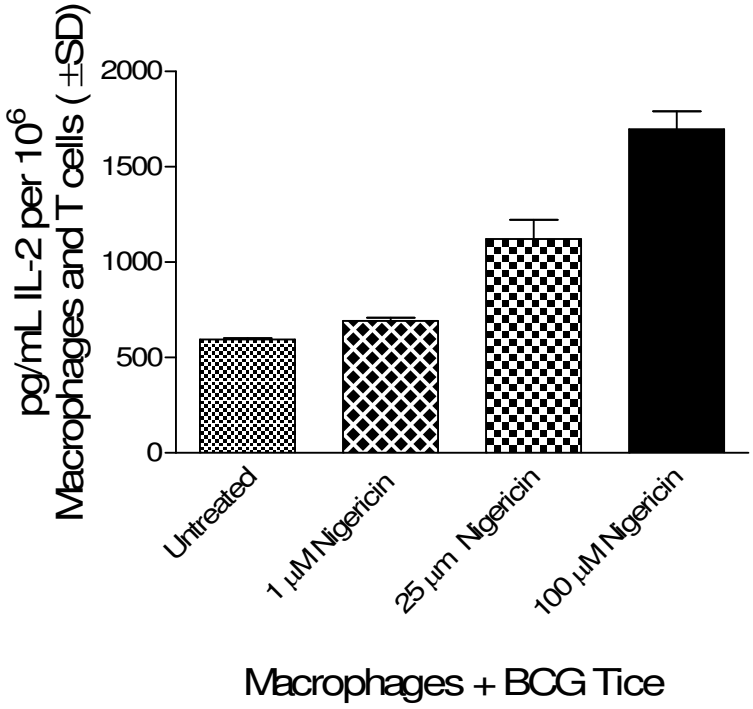


Figure-6: Nigericin, a calcium ionophore that reduces organelle pH in macrophages, enhances antigen presentation in BCG infected macrophages. *Primary murine macrophages were plated into 24 well plates, treated with varying doses of nigericin for 4 hours, infected with BCG Tice for 4 hours, washed and then overlaid with BB7 T cells for IL-2 assay as in Figure-5 (one of 3 similar experiments shown).*

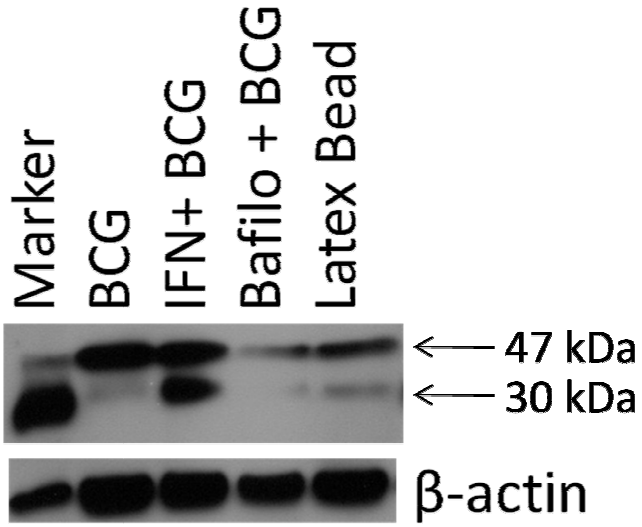


Figure-7: Bafilomycin treatment of macrophages increases the pH of the phagosome lumen and prevents activation of Cathepsin-D. *C57BL/6 derived BMA macrophage cell line was treated with either 100 nM bafilomycin for 18 hrs or 50 ng/mL IFNγ as a positive activator, followed by infection with BCG for 24 hrs. Control phagosomes were latex beads from naïve macrophages. Macrophages were washed and BCG or latex bead phagosomes were purified on sucrose gradients and analyzed using western blot with antibody to Cathepsin-D. Data show that 56 kDa Cat-D inactive form is present in BCG that show enrichment of active 30 kDa band after IFNγ treatment, but remain inactive in bafilomycin treated macrophages. As expected, phagosomes containing latex beads contain active forms of cat-D, even without macrophage activation. One of two separate experiments with similar results is shown.*

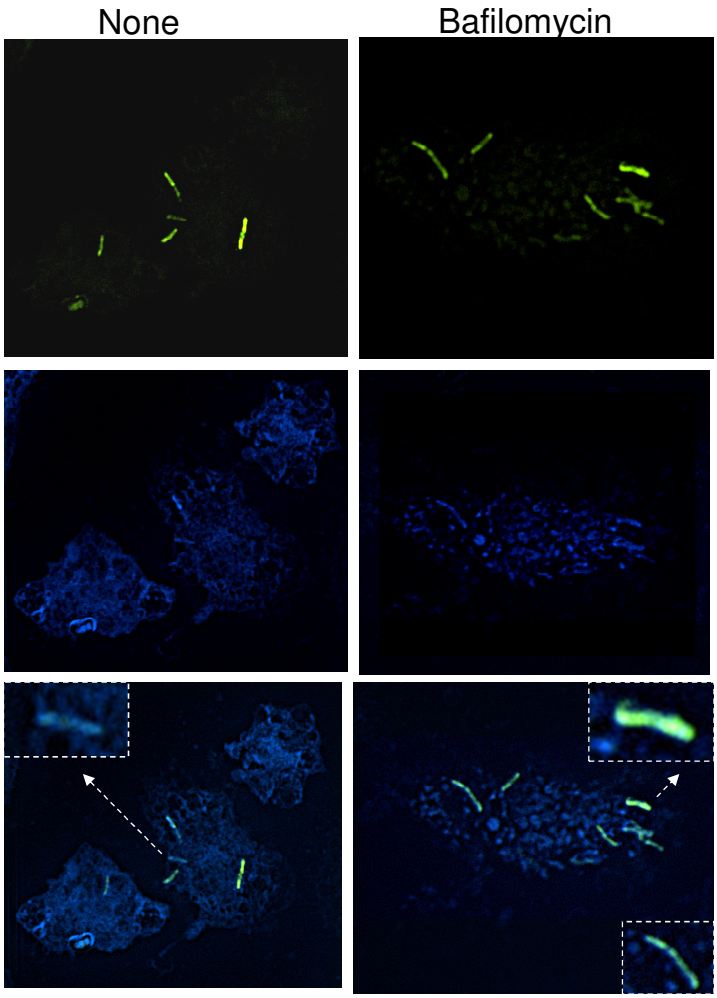


Figure-8A: Bafilomycin treatment increases the pH of the phagosome lumen and prevents acidification. The fluorescent dye Lysosensor yellow-blue dextran remains largely green in gfpBCG phagosomes after bafilomycin treatment. *C57BL/6 derived macrophages were treated with 100 nM bafilomycin (vATPase inhibitor) for 18 hrs followed by 60 min treatment with nigericin and infection with gfpBCG for 4 hrs. Macrophages were washed, incubated in fluormount and phagosomes imaged using a Nikon microscope with metaview software. gfpBCG co-localizing with LSY were scored blue vs. green bacteria and per cent co-localizing gfpBCG determined in 100 macrophages in 2 separate experiments. Quantitation is shown in Figure 8B.*

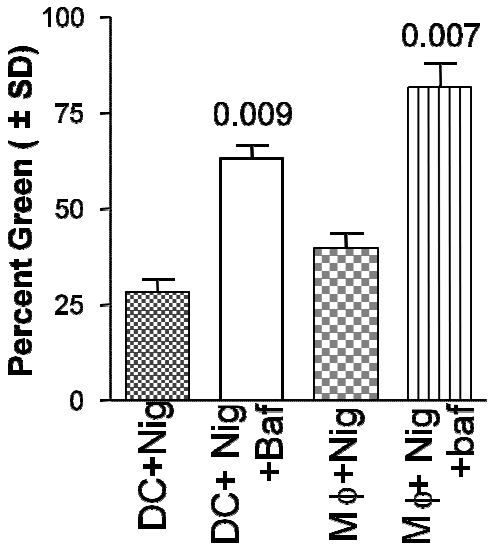


Figure-8B: Bafilomycin treatment increases the pH of the phagosome lumen and prevents acidification. *gfpBCG phagosomes were imaged using a Nikon microscope with metaview software. gfpBCG are generally green since BCG associated phagosomes are near neutral. Prior treatment of macrophages with nigericin makes them acidic, while treatment with bafilomycin makes them neutral. Number of gfpBCG fluorescing with LSY were scored blue (acidic) vs. green (neutral) bacteria and per cent green gfpBCG determined in 100 macrophages of triplicate chambers per experiment in 2 separate experiments (p value determined using t test).Excitation 480 nm and emission 530 nm. LSY dextran was from Invitrogen, Molecular Probes.*

Chapter 2-2

Characterize the BCG phagosome for pH regulating processes that determine the efficacy of antigen production

Sub Aim IB: Investigate whether the phagocyte oxidase (phox) that secretes superoxide into phagosomes affects pH of the phagosome and thereby modulates antigen production

INTRODUCTION

There are several mechanisms that APCs can use in order to defend against invading pathogens. The vATPase induced acidification of macrophages and dendritic cells leads to the generation of active forms of Cathepsin-D, that by itself can be bactericidal as well as proteolytic(36). In addition, a multi-component NADPH oxidase complex (phox) accumulates on phagosome membrane soon after phagocytosis of mycobacteria (77). The phox complex has many subunits, including the major components $p22^{phox}$, $p47^{phox}$, $p67^{phox}$ and $p91^{phox}$ (78-81). Proper assembly of these subunits on the membrane of the phagosome leads to the superoxide-radical O_2^- being pumped into the phagosomes. Superoxide by itself is bactericidal for a variety of microbes but in the presence or iron and halide ions leads to the formation of more bactericidal radicals like hydroxyl radical, H_2O_2 and hypochlorous acid. Superoxide is the lead oxidative radical produced from phox complex and can alter the pH of the phagosome through two mechanisms. In one mechanism superoxide combines with protons pumped in from vATPase, and forms hydrogen peroxide that can neutralize the pH. In a second mechanism it can also spontaneously dismutate into oxygen and water to raise the lumen pH. It has been reported that phox can control the pH of DCs and affect cross-presentation, a major route for MHC-I type antigen presentation (82). However, its role in the regulation of antigen presentation through MHC-II has remained unclear.

HYPOTHESIS

In this chapter, we hypothesized that inhibition of phox activity using the NADPH oxidase inhibitor, diphenylene-iodonium chloride, would reduce pH and lead to enhanced antigen presentation.

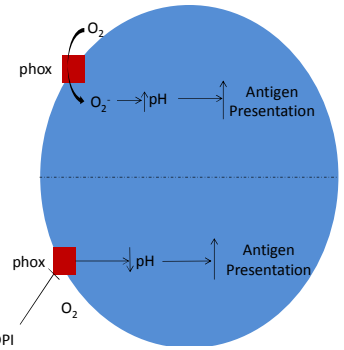


Figure-9: Proposed action of phox on the regulation of pH and the likely effect of DPI

METHODS

C57BL/6 mouse derived macrophages and dendritic cells were purified as per previously published methods (METHODS 5-1). They were incubated with varying doses of the phagocyte oxidase inhibitor diphenylene iodonium chloride (DPI) for 24 hours and infected with BCG vaccine strain for 4 hrs. Monolayers of cells were washed and over laid with BB7 T cells, which release IL-2 when Ag85B epitope is presented from macrophages or DCs to T cells (METHODS 5-2). IL-2 was quantitated using sandwich ELISA.

RESULTS AND DICSUSSION

Figure-10 illustrates that the inhibition of phox complex using DPI led to a surprising decrease in antigen presentation by both DCs and macrophages. We had anticipated in our initial hypothesis that phox secreted superoxide would either neutralize the protons being pumped in by vATPase or would rapidly dismutate into oxygen and water. Either of these events would lead to a neutral pH inside phagosome. Thus, inhibition of the phox enzyme through DPI was expected to reduce the pH of the phagosome and lead to enhanced antigen presentation through activation of enzymatically active Cathepsin-D. The fact that DPI treatment of macrophages and DCs reduced antigen presentation suggests that the pH of the BCG phagosome was not rendered acidic. Although not anticipated, our observation appears inconsistent with the recent report that inhibition of the phox through DPI does not significantly affect the phagosomal pH, when latex beads are used to measure the pH of the phagosomes in macrophages (83). However, we used live BCG as a phagosome in contrast with inert latex beads in macrophages, and it was possible that the bacterial derived factors

could change the intracellular pH. We therefore propose that BCG derived superoxide dismutase (SOD), which dismutates superoxide anion, interacts with other oxidants within the phagosome, eventually raising the pH, despite ongoing acidification of the lumen through vATPase. That, the BCG phagosomes are near neutral was consistent with this hypothesis (36).

We conclude therefore that both vATPase and phox pumps can alter the pH of the phagosome while BCG secreted SOD enzyme also perhaps plays a significant role in the modulation of lumen pH.

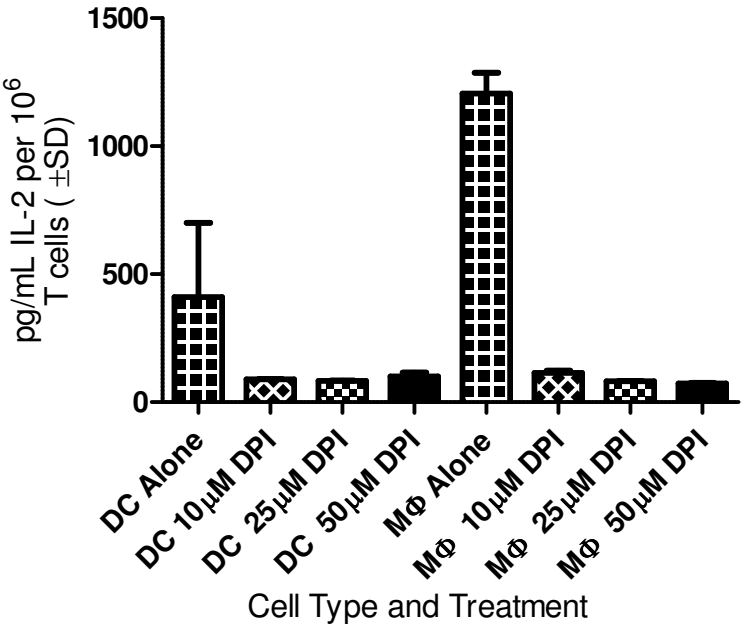


Figure-10: Inhibition of phagocyte oxidase in macrophages and DCs with diphenylene-iodonium chloride leads to decreased antigen presentation. *Macrophages and DCs were incubated with varying doses of DPI for 4 hrs followed by infection with BCG for another 4 hrs (MOI 1:1). Washed monolayers were overlaid with BB7 T cells and IL-2 release measured after 18 hrs (Mean± SD; one of 2 separate experiments with similar data shown; all values for DPI treatment are significant compared to untreated)*

Chapter 2-3

Characterize the BCG phagosome for pH regulating processes that determine the efficacy of antigen production

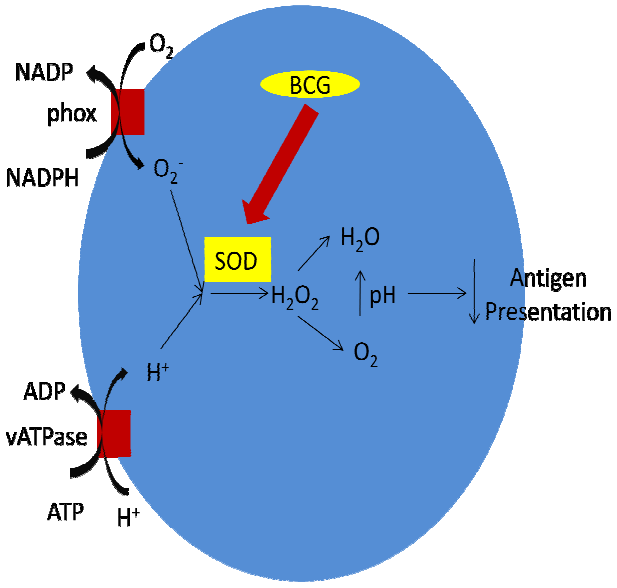
Sub Aim IC: Determine if phagosome pH can be modulated through the use of SOD mutants of BCG vaccine

INTRODUCTION

Data shown in chapters 2-1 and 2-2 showed that blockade of vATPase and phox enzymes led to inhibition of antigen production thereby indicating that pH determines the level of antigen production. Thus, there are at least two regulatory mechanisms that control the pH of the phagosome and affect the antigen production. However, these events excluded the role played by the BCG vaccine itself, since BCG secretes a powerful enzyme superoxide dismutase (SOD) that dissociates superoxide into oxygen and water. Under physiological circumstances, BCG infected macrophages show minimal amounts of Ag85B being produced and presented to T cells, which we hyppthesize is because of a neutral pH (36). We therefore speculated that under natural circumstances of infection of macrophages, BCG secreted SOD was inactivating superoxide from phox enzyme and vATPase was not sufficient to acidify the lumen. In this study, we examined the role of SOD.

HYPOTHESIS

In this chapter, we hypothesized that BCG induced SOD may play role in determining intra-phagosomal pH perhaps by dissociating superoxide leading to molecular oxygen and water. We also speculated that molecular oxygen may neutralize protons coming from the vATPase pump, so that the net effect of SOD is neutralization of the pH of the



us Superoxide
I which decreases
eby antigen

phagosome lumen. We therefore hypothesized that a BCG vaccine candidate that does not secrete SOD or is defective in secreting SOD should be unable to modulate pH of the phagosome and thereby adversely affect antigen production.

SUPEROXIDE DISMUTASE

Mycobacteria contain genes encoding for superoxide dismutase (SOD)(70,71). Superoxide dismutase is a secreted enzyme that spontaneously dismutates into molecular oxygen and water (hence the name). SOD is an essential gene in mycobacteria and can only be suppressed through genetic manipulation strategies. BCG strains lacking the ability to produce or secrete SOD were formed from a parent BCG-Tice strain (a kind gift of Doug Kernodle Ph.D., Vanderbilt University). Three mutants from a parent strain of BCG-Tice were made, BCGΔ*SODA*, BCGΔ*SecA2* and BCGΔ*SODASecA2*. These strains are respectively, BCG that has SOD suppressed, BCG that has the secA apparatus disrupted and BCG that has both SOD suppressed and SecA2 disrupted. SecA is required for secretion of SOD from mycobacteria, and therefore knocking out this gene leads to a lack of secretion of SOD from BCG into the lumen of phagosomes. These strains were constructed by inserting a hygromycin resistance vector (*hygR*) into the genes encoding SOD or SecA, thereby inactivating the target gene (70,84,85).

METHODS

Macrophages and DCs were infected with wild type Tice BCG and the mutants and analyzed for the induction of IL-2 using the standardized antigen presentation system (METHODS 5-2). Macrophages were also infected with Tice and mutant strains and

phagosomes of the bacteria purified using sucrose gradients (METHODS 5-7). The phagosomes were analyzed for the content of Cathepsin-D protease to determine if the phagosomes were acidic enough to generate active forms of Cathepsin-D (METHODS 5-3 and 5-4). Furthermore, C57BL/6 mice were immunized subcutaneously with BCG and an SOD-deficient mutant strain, and 2 weeks later, the spleen T cells were analyzed for $CD4^+$ T cells secreting IFNγ using flow cytometry.

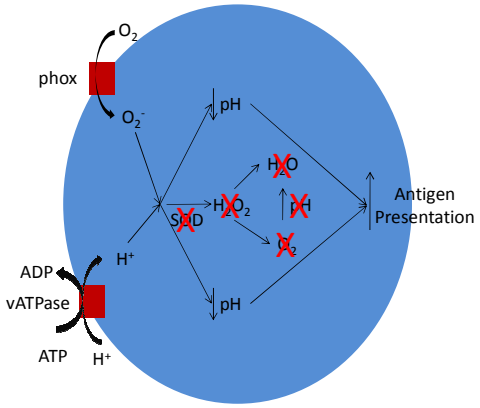
RESULTS AND DISCUSSION

We demonstrated earlier that preventing acidification of the phagosomes through bafilomycin treatment inhibits antigen production. Paradoxically, inhibition of phagocyte oxidase activity via DPI also decreased antigen presentation from BCG vaccine infected macrophages. These data together suggest that pH is a critical determinant of the phagosome that determines the antigen production. It is obvious that enhancing antigen production will make a stronger BCG vaccine, but it is difficult to modulate the pH of the macrophage or phagosome pH through chemical intervention without causing toxicity. In this study, we used genetically modified mutants of BCG Tice strain BCGΔ*SODA*, BCGΔ*SecA2* and BCGΔ*SODASecA,* to infect the macrophages. The hypothesis was that, BCG strains secrete copious amounts of SOD enzyme which can detoxify the superoxide and enhance phagosome pH. Mutants that have reduced SOD would be unable to modify pH and would therefore be more susceptible to antigen processing and presentation.

Figure 13 illustrates that the SOD mutants were indeed processed better by the macrophages and DCs, which presented Ag85B better, with elevated IL-2 levels. In order to determine if

Figure-12: Loss of SOD increases antigen presentation by macrophages and DCs.

SOD altered the pH of the phagosome and affected proteolysis, the phagosomes of wild type Tice and mutants were then purified from macrophages and analyzed for the enzyme Cathepsin-D using western blot. In our previous studies, we demonstrated that Cat-D was the major protease that produced Ag85B for presentation to BB7 T cells. In this study (Figure 14), the active form of Cat-D was enriched within the phagosomes of the mutants BCGΔ*SODA* and BCGΔ*SecA2* compared to the wild type Tice phagosomes. This implied that antigen production by macrophages with the latter mutants correlated with the presence of active Cat-D (30 kDa). Since the generation of active Cat-D (30 kDa) from immature Cat-D (56 kDa), is a pH dependent process (36), this suggests that the SOD enzyme from BCG directly affects the pH of the phagosomes and affects levels of active Cat-D. The ability of SOD mutants to generate better immune responses at the level of macrophages was also reflected in mice. Mice immunized with SOD mutants were found to respond with increased numbers of IFNγ positive $CD4^+$ T cells through Elispot analysis (Figure 15).



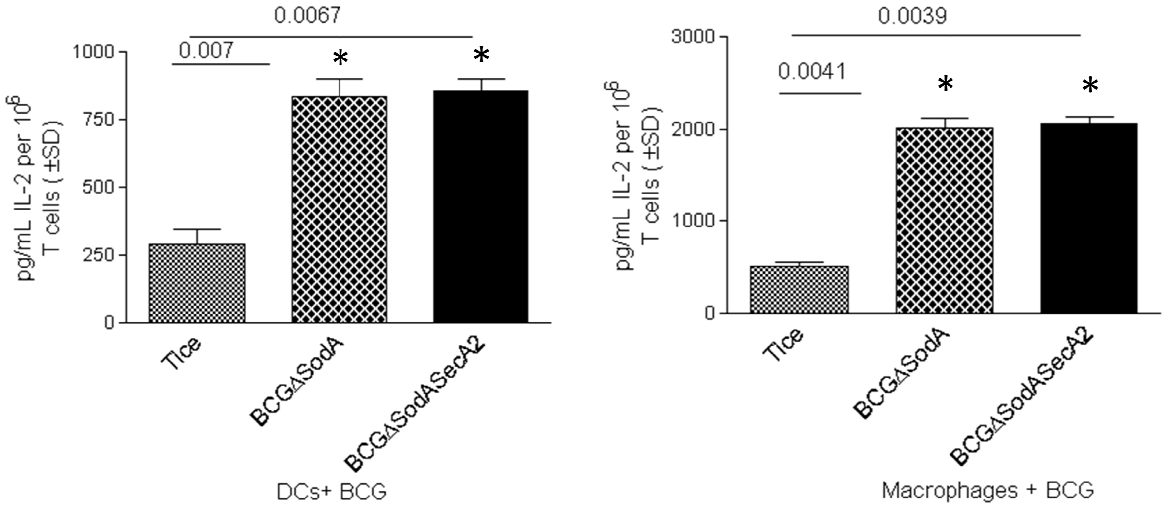


Figure-13: The superoxide dismutase (SOD) defective mutant strains of BCG Tice sub strain *(BCGΔSODA, and BCGΔSODASecA2)* ***are more effectively processed by macrophages to result in enhanced Ag85B presentation to T cells in vitro.*** *Macrophages or DCs from C57BL/6 mice were infected with either wild type Tice or mutant strains at an equal MOI (1:1), incubated for 4 hrs, washed and overlaid with BB7 T cells for 18 hr. IL-2 was quantitated using sandwich ELISA (one of four similar Elisa experiments shown). P values obtained by using student t test.*

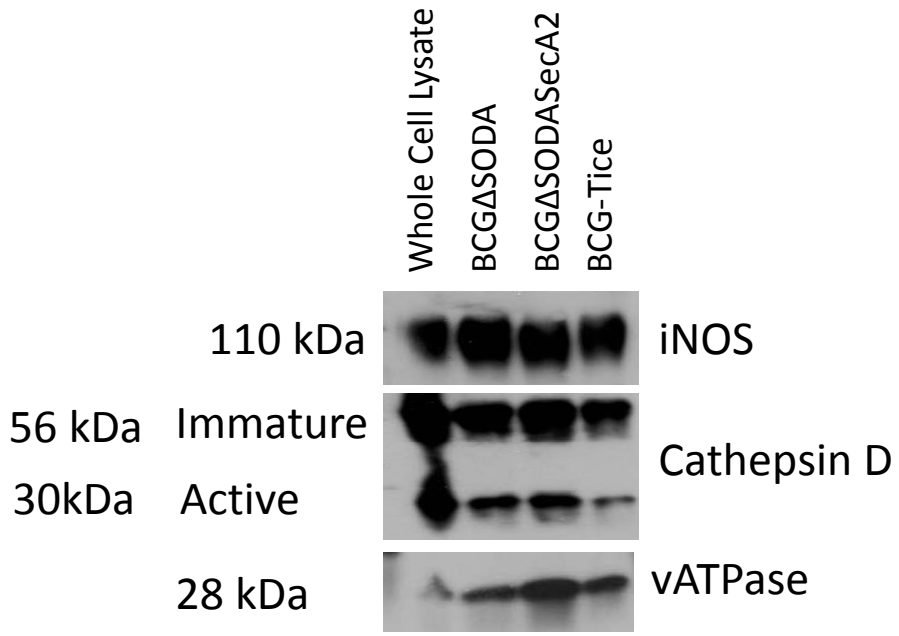


Figure-14: The phagosomes of superoxide dismutase (SOD) defective mutant strains of BCG Pasteur sub strain Tice, *(BCGΔSODA, BCGΔSODASecA2)* ***contain the more mature form of Cathepsin-D.*** *Macrophages from C57BL/6 mice were infected with either wild type Tice or mutant strains at an equal MOI (1:1), incubated for 24 hrs, washed and phagosomes purified using sucrose gradients. Equal amounts of proteins were loaded onto a 10% SDS gel and blotted. Bands were then analyzed for Cathepsin-D using western blot with antibody to Cat-D and antibodies to vATPase and iNOS as positive controls. One of two separate similar experiments is shown.*

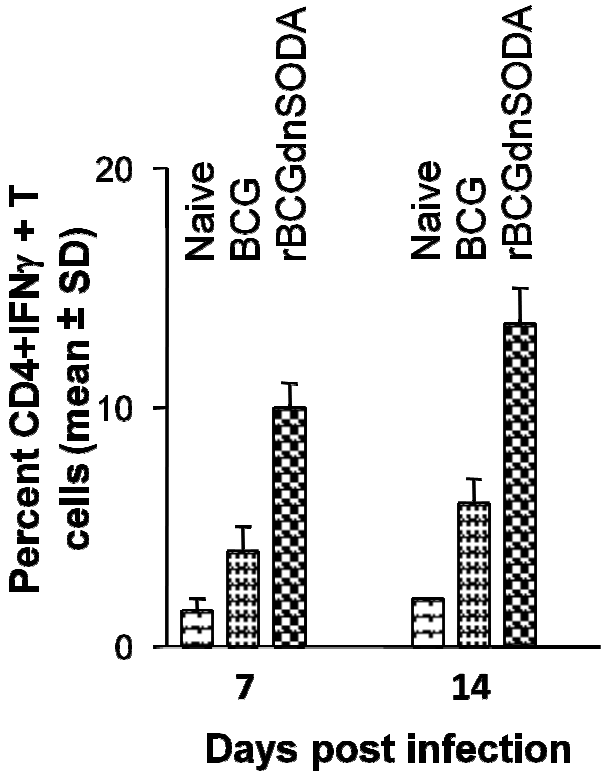


Figure-15: The SOD defective mutant strain of BCG sub strain Tice (BCGdnSODA) is more immunogenic in mice. *C57BL/6 mice were infected with wild type Tice or SOD mutant strain at 10^6 CFU per subcutaneous dose and on 7 and 14 days after infection, splenic T cells were assayed for IFNγ secreting $CD4^+$ T cells using flow cytometry. Data show that the mutant strain induces a stronger $IFN\gamma^+$ $CD4^+$ T cell response than wild type Tice. One of two separate but similar experiments is shown.*

CHAPTER-2

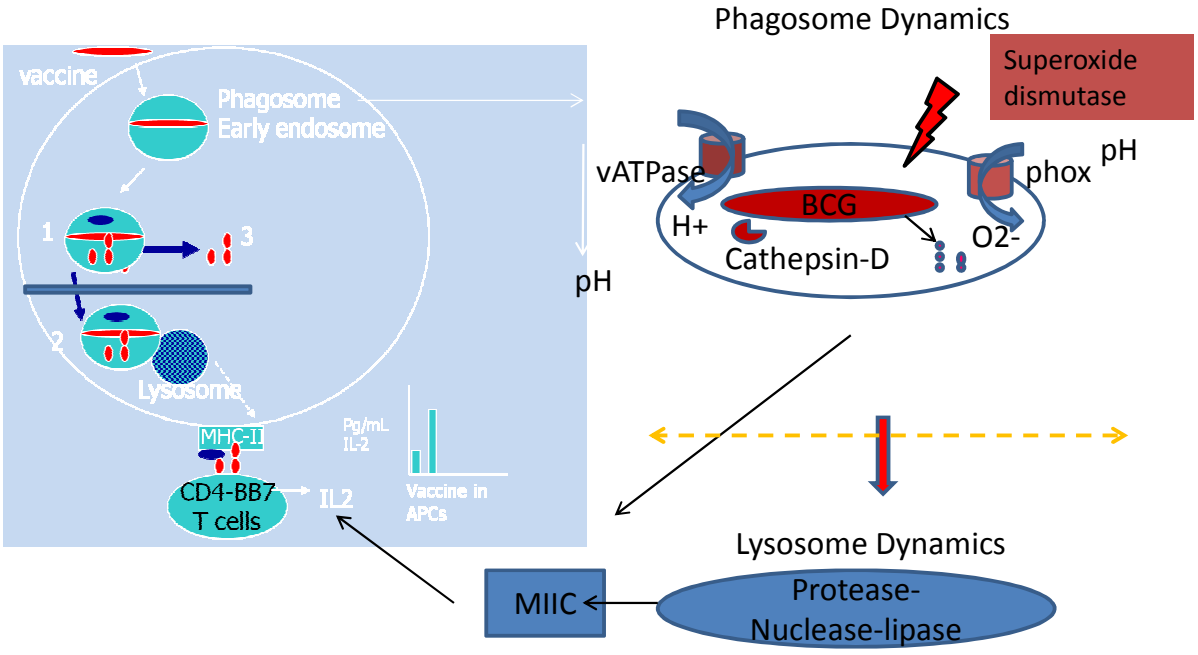
GENERAL DISCUSSION

Role of pH as a regulatory mechanism for antigen production

The phagosomes of BCG vaccine within macrophages and DCs seem to be regulated by three separate, but overlapping mechanisms that affect the overall pH of the lumen. Two of these mechanisms are innate mechanisms of the host macrophages, namely vATPase that assembles as a multimeric enzyme on phagosomes to acidify, and the multimeric phagocyte oxidase that also assembles on the phagosome, when BCG vaccine invaginates into the plasma membrane forming the phagosome. While vATPase acidifies, phox enzyme alkalinizes the lumen and thus the net pH of the lumen appears to be determined by the bacterially derived enzyme SOD. SOD has been thought to be an immune-evasive factor secreted by BCG vaccine that detoxifies the superoxide and in doing so causes the pH to again become neutral. It is noted here that there are many other pumps that can affect the phagosome pH and this is a simplified scenario of the complex environment. Blockade of phox utilizing DPI was able to markedly inhibit antigen presentation, and it is possible that there are other factors at play. For example, investigators argue that oxidants inactivate cathepsins (86) Nevertheless, disruption of the SOD enzyme in the BCG vaccine led to an enhanced immunogenic phenotype of BCG vaccine. This argues that the lumen pH may well be regulated by vATPase and phox but bacteria like BCG also seem to have evolved to cause immune evasion through SOD. BCG is therefore able to overcome the effects of vATPase and phox. Together, these data support our contention that an enhanced

understanding of the pH regulatory mechanisms of the phagosomes will enable us to design better vaccination strategies for tuberculosis.

Figure-16: *Mechanisms of pH regulation in phagosomes affect antigen production in macrophages and dendritic cells.*



CHAPTER 3

Aim II

Utilize proteomics to identify novel mechanisms that may mediate antigen processing of the BCG vaccine

CHAPTER 3-1

Utilize proteomics to identify novel mechanisms that may mediate antigen processing of the BCG vaccine

Sub Aim IIA: Characterize the proteins of BCG vaccine phagosomes through proteomics

INTRODUCTION

When latex beads or bacteria are internalized into macrophages they are enclosed within a membrane bound compartment called the phagosome(52,53). Desjardin group did a comprehensive analysis for the proteins associated with the latex bead phagosome, and found numerous proteins associated with the phagosome, many with undefined functions (87,88). Since particles phagocytosed by macrophages and DCs are destined for degradation in lysosomes, the phagosomes move through successive stages to lysosome in a process called maturation or phagosome-lysosome fusion. This process is regulated in turn by a series of proteins like rabs and SNARES (89-91).

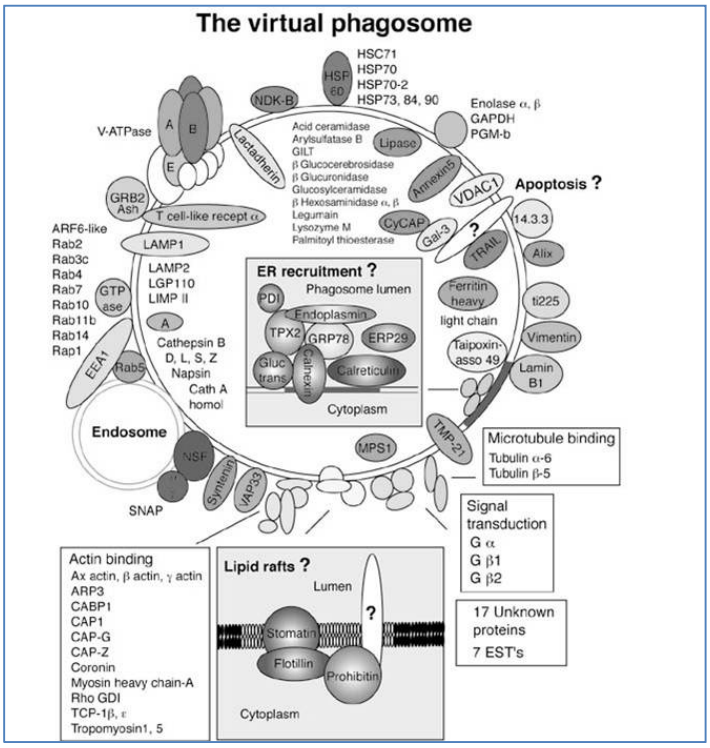


Figure-17: Virtual phagosome demonstrating numerous proteins on phagosome surfaces. http://www.dsv.cea.fr/thema/lcp/phagosome/phago2_fr.htm

Compared to the phagosomes containing latex beads, the bacterial phagosome is more complex because host interaction with bacteria recruits more proteins into the phagosome. While the phagosome can potentially contain hundreds of proteins, identifying all the proteins associated with the bacterial phagosome and differentiating them on the basis of host and bacterial origin is cumbersome using traditional techniques such as western blotting. Our collaborator, Dr. Qing Bo Li – University of Illinois at Chicago, developed an innovative process of peptide mapping using proteomics. In this process phagosomes

purified from macrophages are subjected to two dimensional gel electrophoresis and spots are excised and subjected to Mass spectrophotometry analysis. Proteins are identified on the basis of their amino acid sequence and quantities compared using a software program. These methods have been published (92-94).

HYPOTHESIS

Since we found that BCG vaccine phagosomes had a near neutral pH and three mechanisms regulated the internal pH, we hypothesized that perhaps the phagosome also contains additional proteases that mediate generation of peptides leading to generation of immune responses.

METHODS

We previously published methods to purify mycobacterial phagosomes from mouse macrophage cell lines (36). In this study, we infected the C57Bl/6 mouse derived BMA.A1 macrophage like cell line with BCG vaccine and wild type Mtb H37Rv, in addition to ΔfbpA mutant, a novel lysosome-fusion competent vaccine comparable to BCG (95) (Figure-18). After 24 hrs of infection, the phagosomes were purified using sucrose gradients and shipped to the laboratory of Dr. Li, UIC, where, a proteomic analysis was performed. The data were analyzed and presented in a graphical format (additional detailed procedures are outlined in METHODS 5-9).

RESULTS AND DISCUSSION

The proteomic analysis found over three-hundred proteins associated with each of the three species of mycobacterial phagosomes. Statistical analysis was performed to compare the proteins from purified phagosomes containing one of three mycobacterial strains (BCG, *ΔfbpA* and wild type H37Rv). We first determined that proteins (e.g., rabs) that are known to be associated with endosome-phagosome traffic were present in phagosomes. Interestingly, several proteins of hitherto undefined function were also differentially enriched on the phagosomes. These are shown as the three distal groups in Figure-19 and the putative functions of these proteins are reproduced from the literature in Table-I. We observed that Nicastrin was enriched on Mtb H37Rv phagosomes compared to BCG and ΔfbpA. However, Nomo, a protein that associates with nicastrin was more enriched on BCG phagosomes (96-98). Although subtle variations in the quality of phagosomes prepared could affect the relative amounts of proteins on phagosomes, it was interesting to find that of the 300 or so proteins on phagosomes, components with protease activity like nicastrin were enriched. Thus, proteomic analysis identified certain proteins with protease function in mycobacterial phagosomes. This assumed significance since previous reports indicated that nicastrin typically associates with two other components, APH and presenilin to form the γ-secretase complex. The γ-secretase complex is a membrane-associated protease and suggested to recycle and remodel phagosomes (99). The data suggested a novel hypothesis, that nicastrin and associated proteins could play a proteolytic role associated with the phagosomes.

Proteomic analysis

- **BCG vaccine** -Pasteur
- Wild type M.tuberculosis H37Rv
- ΔfbpA strain-KO strain

Macrophages (BMA cell line from C57Bl/6 mice) were infected with H37Rv, ΔfbpA or BCG-Pasteur

↓

Phagosomes collected and purified

↓

Samples frozen in SDS sample buffer and shipped to UIC, Chicago

↓

Mass spectroscopy analysis performed, data tabulated and statistical analysis performed

BMA cell line: Kovacsovics-Bankowski M, Rock KL. Science. 1995 Jan 13;267(5195):243-6.

Figure-18: *Outline of the procedure used to purify the phagosomes which were then subjected to proteomic analysis (100)*

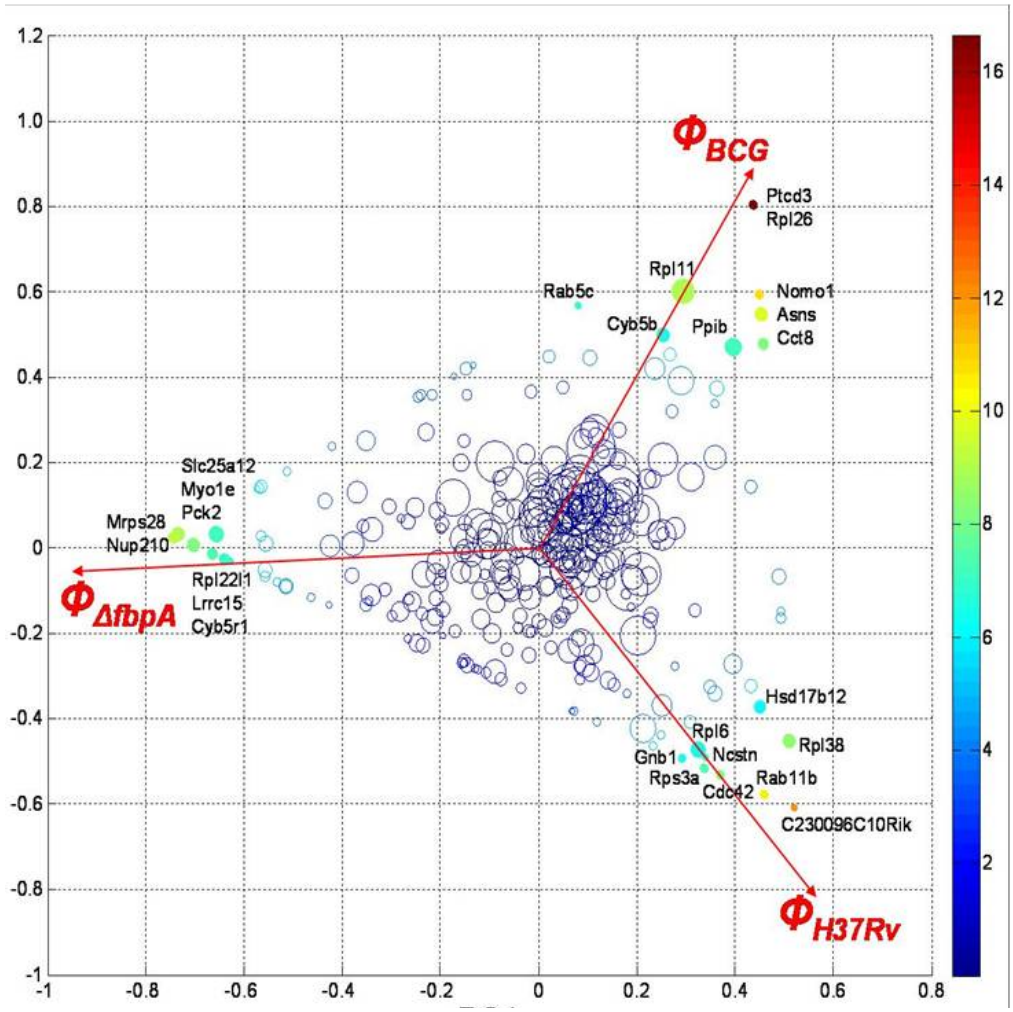


Figure-19: Scatter plot of the T2 values of over 300 proteins quantified in the phagosomes (Φ) of the BCG-, ΔfbpA-, and Mtb H37Rv-infected macrophages*. The x,y,z axis represent the different strains of mycobacteria utilized for this experiment. Each circle represents a protein, and the size of the circle represents the abundance of the protein on the phagosome . The color indicates the fold change of the protein over the phagosomes of the other strains. The marker size is proportional to the square-root of the averaged (5 repeats of each phagosomal sample) intensity of that protein in the three phagosome samples as determined by mass spectroscopy (reproduced from Li et al, (92)).*

Table-II: Proteins relatively enriched on BCG phagosomes are noted with putative function identified from protein data base. *There were two studies published, from which the enriched proteins were identified (92-94,101).*

Proteins enriched on BCG phagosome	This study	Lee et al (134)
asparagine synthetase	+	
chaperonin containing TCP1	+	
Cytochrome B5	+	
Nicastrin	+	+
Nomo (pM5)	+	
peptidylprolyl isomerase B (cyclophilin B)	+	
Rab 5	+	+
Ribosomal Component Proteins	+	+

Nicastrin binding partner

Recycling nutrients

Chaperone for vATPase
PLoS One. 2010 (11):e13930

CHAPTER 3-2

Utilize proteomics to identify novel mechanisms that may mediate antigen processing of the BCG vaccine

Sub Aim IIB: Compare the proteomics of BCG phagosomes derived from macrophages and dendritic cells

INTRODUCTION

While macrophages and dendritic cells are both capable of processing and presenting peptides to $CD4^+$ and $CD8^+$ T cells, it has been known that only DCs are able to cross-talk with naïve T cells and prime them for antigen specificity and expansion into effecter T cells(49,50,52,53). We wondered whether phagosomes of DCs and macrophages are comparable. Since we identified in our previous chapter that some proteases were enriched in the mycobacterial phagosomes, we sought to understand whether similar proteins were present in phagosomes purified from DCs. In this study, we hypothesized that phagosomes derived from macrophages and DCs were similar in protein content, at least with respect with some proteases.

METHODS

Methods were similar to those described in the previous chapter but the phagosomes were fractionated from CY-15, a dendritic cell line provided by Dr. Blankenstein (102). For comparison, phagosomes of three species of mycobacteria (BCG, wild type H37Rv and *ΔfbpA*) were purified at the same time from BMA.A1 macrophage cell line. All infections and incubation conditions were identical, other than the cell line.

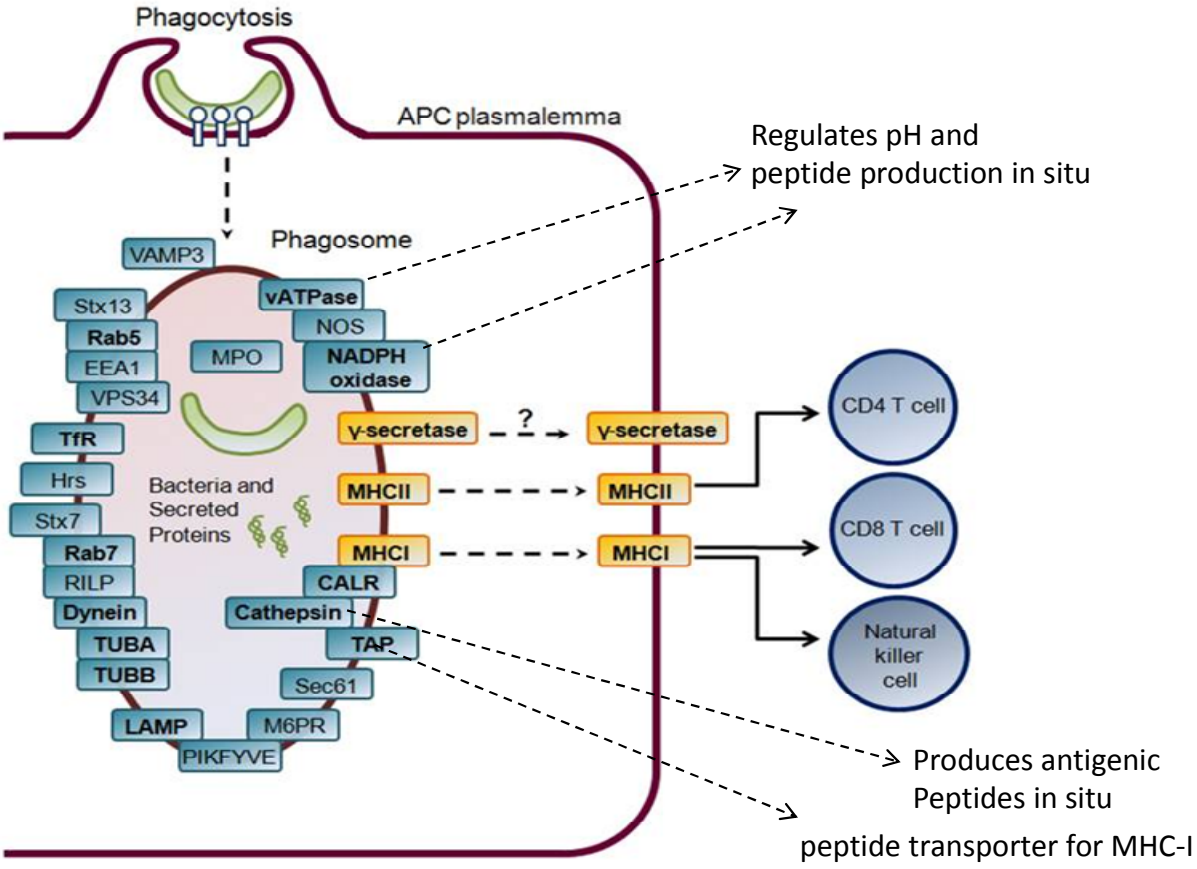
RESULTS AND DISCUSSION

DC dericed phagosomes containing BCG, wild type H37Rv and *ΔfbpA* were analyzed and found to contain proteins largely similar to those found in macrophages**.** Table-III & Figure-20 illustrate proteins that were commonly found among phagosomes from two cell lines. It was noted that nicastrin was again found to be enriched on the mycobacterial phagosomes of DCs.

Table III: *An abridged list of proteins enriched on the phagosomes containing BCG vaccine, wild type M.tuberculosis (shown as Rv) and ΔfbpA mutant (designated as KO) analyzed using proteomics. The abundance of nicastrin is highlighted. As in Figure-19 over 350 proteins were analyzed and the numbers indicate the relative expression of proteins associated with phagosomes containing various strains of mycobacteria and within either dendritic cells (DCs) or macrophages (MPs).*

					In MPs			In DCs			In MPs vs in DCs			
Gene symbol	Protein description	Phagosome pathway components	Spectral counts	Peptide counts	$\Phi_{MP,KO}/\Phi_{MP,Rv}$	$\Phi_{MP,KO}/\Phi_{MP,BCG}$	$\Phi_{MP,Rv}/\Phi_{MP,BCG}$	$\Phi_{DC,KO}/\Phi_{DC,Rv}$	$\Phi_{DC,KO}/\Phi_{DC,BCG}$	$\Phi_{DC,Rv}/\Phi_{DC,BCG}$	$\Phi_{MP,KO}/\Phi_{DC,KO}$	$\Phi_{MP,Rv}/\Phi_{DC,Rv}$	$\Phi_{MP,BCG}/\Phi_{DC,BCG}$	
Msr1	macrophage scavenger receptor 1	Scavenger receptors	2	1	3.3	**8.8**	5.6	-0.7	-1.9	-1.2	**8.9**	4.9	-1.8	0.2
Ncstn	Nicastrin	gamma-secretase	4	1	3.1	**5.0**	1.9	-2.8	0.1	2.9	**5.4**	-0.5	0.5	**4.8**
Rab5c	RAB5C, member RAS oncogene family	Rab5	33	3	0.6	0.0	-0.5	**2.2**	**3.0**	0.8	**-1.8**	-0.1	1.2	**-1.7**
Rab7	RAB7, member RAS oncogene family	Rab7	121	5	0.5	0.0	-0.5	0.4	-0.1	-0.5	**1.0**	0.9	0.9	1.3
Ncf1	neutrophil cytosolic factor 1	NADPH oxidase	9	2	0.0	-0.9	-1.0	**2.6**	**3.4**	0.9	-1.7	0.8	**2.6**	3.1
Rac1	RAS-related C3 botulinum substrate 1		76	6	**-0.7**	0.0	0.7	**5.4**	**4.3**	-1.2	**-2.4**	3.8	1.9	1.3
Sec22b	SEC22 vesicle trafficking protein homolog B (S. cerevisiae)	Sec22	25	3	**5.1**	0.1	**-5.0**	1.6	2.8	1.3	1.8	-1.7	**4.5**	3.0

Figure-20: Proteins enriched on the phagosomal membranes of mycobacterial phagosomes (BCG vaccine, M. tuberculosis and ΔfbpA mutant) are illustrated. *The proteins were identified from a more exhaustive list of proteins analyzed on phagosomes of mycobacteria, illustrated in the Table II. Putative functions are highlighted with arrows.*



CHAPTER 3-3

Utilize proteomics to identify novel mechanisms that may mediate antigen processing of the BCG vaccine

Sub Aim IIC: Investigate the function of target protein (nicastrin) identified through proteomics

INTRODUCTION

Tuberculosis is the leading cause of death due to an infectious disease and BCG is the only approved vaccine for humans. BCG gives variable protection against tuberculosis depending upon geographic regions and the variation has been thought to be due to the loss of some immunogenic antigens, strain variation and population immunity factors. In addition, earlier studies show that the BCG vaccine sequesters within early endosomes, which do not fuse with lysosomes (103). Since peptide processing and presentation through MHC-II is essential, this suggested that antigen presenting cells (APCs) like macrophages and dendritic cells would be deficient in presenting MHC-II dependent peptides from BCG. Indeed, phagosomes of BCG were near neutral in pH due to interplay of complex factors involving vATPase and phagocyte oxidase enzyme and as a consequence, intra-phagosomal cathepsin-D was inactive (36). Furthermore, using a T cell hybridoma specific for a major secreted antigen from BCG antigen 85B, we found that APCs infected with BCG showed a decreased antigen presentation (36). Curiously, the wild type *Mycobacterium tuberculosis* (Mtb) was the first pathogen reported to evade phagosome-lysosome fusion in macrophages (37-39). It is now evident that BCG vaccine also has immune evasion mechanisms, that enable the bacterium to be sequestered within non acidic phagosomes (104-106) (Chapter 1).

Tuberculosis is an important disease for which efficient vaccination strategies have been sought. We therefore initially analyzed alternative mechanisms of antigen processing within macrophages that facilitate immune recognition of the vaccine or wild type pathogen. We analyzed the proteome of the phagosomes of BCG as well as the wild type Mtb H37RV, purified from mouse macrophages (Chapter 3.1, 3.2). We found that nicastrin, a component

of the enzyme γ-secretase, was enriched on the phagosomes of the wild type Mtb and to a lesser extent on BCG vaccine phagosomes.

γ-SECRETASE

γ-secretase is a multi-subunit membrane-associated enzyme complex known to participate in Notch signaling and cleavage of amyloid precursor protein (APP). The complex consists of nicastrin, presenilin and APH (98,107,108). Previous studies have focused on the role of γ-secretase during Alzheimer's disease; γ-secretase cleaves the Amyloid Precursor Protein (APP) and the byproduct, amyloid β (Aβ), is the primary component of amyloid plaques that cause the disease(109,110). γ-secretase cleaves the transmembrane portion of APP, producing the 4 kDa Aβ peptide, which is implicated in the progression of Alzheimer's disease.

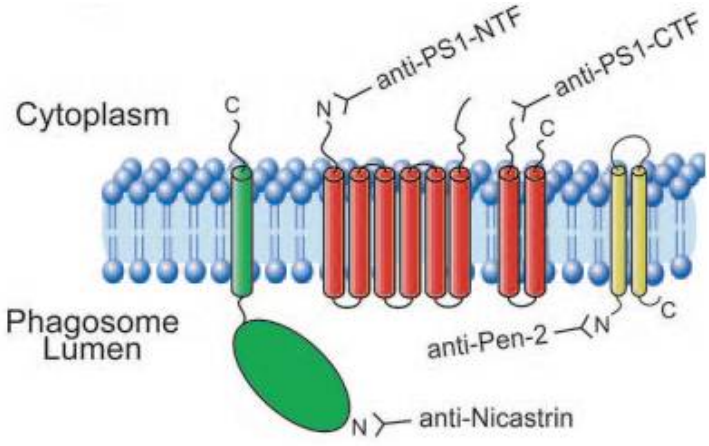


Figure-21: Membrane structure of γ-secretase (97)

γ-Secretase has also been implicated during Notch signaling, a critical pathway of cell development (111,112). Notch signaling involves activation and internalization of Notch receptors, which bind to ligands. Once internalized, the ligand-receptor complex is

endocytosed and the complex is cleaved by γ-secretase, releasing a signaling peptide that translocates to the nucleus for activation of target genes. Finally, a recent study has demonstrated that γ-secretase is a component of the phagosome, and proposed that it may remodel the phagosome and recycle its components. In addition, γ-secretase is up-regulated by IFN-γ while, two additional substrates of γ-secretase, CD-44 and CD-91 are known to be involved with phagocytosis (112).

HYPOTHESIS

Previous chapters (Chapter 3.1,3.2) demonstrated that nicastrin and Nomo are associated with mycobacterial phagosomes. Interestingly, BCG vaccine secretes a number or proteins including Ag85A and B into the phagosome lumen. Since nicastrin associates with APH and Presenilin to form the γ-secretase complex, which is a membrane associated protease, we speculated that nicastrin or γ-secretase components can fortuitously cleave BCG vaccine derived proteins. Thus, we hypothesized that γ-secretase complex may generate immune responses.

METHODS

C57BL/6 mouse bone marrow derived primary macrophages and DCs were cultured and infected with BCG (Pasteur) or wild type *M. tuberculosis* as previously described (METHODS 5-2). They were overlaid with BB7 T cells specific for Ag85B (kind gift of Dr. Harding, CWRU) and IL-2 secreted in response to Ag85B was quantitated using ELISA. siRNA blockade of γ-secretase was carried out using specific siRNAs obtained from Santa Cruz Biotechnology against nicastrin, APH and presenilin and the control was a scrambled

siRNA supplied also by the manufacturer. Chemical inhibition of γ-secretase was carried out using L-685458.

Immunoflourescence analysis for γ-secretase and localization on BCG phagosomes

APCs were infected with *gfp*BCG or *gfp*Mtb (H37Rv), washed, fixed, counterstained and scored for immunofluorescence co-localization with antibodies directed against the components of the γ-secretase as per published procedures (2) (8)(113,114) (METHODS 5-6).

Membrane or cytosol localization for Nicastrin

Macrophages and DCs were either left naïve or treated with L685458 in 24 well plates, infected with BCG and washed. Monolayers were washed with PBS, extracted with 0.05% digitonin, 0.5% sucrose in PBS-EDTA (DSPE) buffer for 15 min to obtain cytosol fraction, which was then ultra-filtered and stored. The monolayer was washed with cold DSPE three times and the membrane fraction solubilized with 0.1% tritonX-100 in PBS for 15 min and stored until western blot analysis.

Mouse experiments

BM derived, CD11c bead purified DCs were infected before or after treatment with L685,458 (1 μg/mL and 10 μg/mL for 4 hrs) with BCG, washed and 10^6 DCs per mouse injected i.p. into C57BL/6 mice, using 4 mice per group. Untreated DCs injected into mice were negative controls. After 7 days, spleens were removed and IFNγ^+ $CD4^+$ T cells were detected using flow cytometry for intracellular cytokines and by ELIPOST analysis with

Ag85B re-stimulation (8). All animal procedures were conducted under approved institutional protocols.

RESULTS AND DISCUSSION

γ-secretase components are enriched on mycobacterial phagosomes: We previously reported that Nicastrin is enriched on the phagosomes of wild type strain of Mtb (H37Rv) using Mass-spectrometry analysis of purified phagosomes from mouse macrophages (Chapters 3-1, 3-2). To determine whether all three γ-secretase components occurred uniformly on BCG, APCs were infected with *gfp*BCG and stained for nicastrin, APH and presenilin using *in situ* immunofluorescence and followed by analysis of co-localization patterns. Figure 22 illustrates that all three components of γ-secretase were present and co-localized with *gfp*BCG phagosomes, although, rather more intense staining was obtained with nicastrin. Western blot profiles confirmed that the three components of γ-secretase were present on BCG phagosomes and they could be specifically knocked-down using siRNA (Figure 23). In addition, BCG phagosomes purified from macrophages using sucrose gradients had enzymatically active γ-secretase, since they cleaved the amyloid precursor protein into an 87 kDa product (Figure 24).

Membrane localization of nicastrin: Phagosomes intensely stained with antibody against nicastrin with very little cytosolic localization (Figure 22). To further confirm its presence on phagosomes, macrophages were infected with BCG and membrane or cytosol fractions were performed using digitonin extraction of cytosol and triton-X solubilized, cytosol free

membranes as described earlier (METHODS 5-11). Nicastrin was exclusively localized to membrane fraction in the presence or absence of L685,458 (Figure 25)

γ-secretase components process phagosome derived proteins and thus affect antigen production: Since the proteolytic activity of γ-secretase was known and it was enriched on inert latex bead phagosomes, it was proposed that it may be involved in recycling and remodeling phagosome proteins. Interestingly, mycobacteria like BCG vaccine and wild type Mtb secrete Ag85B that accumulates within the phagosomes (115,116). Ag85B has a major T cell epitope and is a powerful immunogen for mice and humans. This epitope is recognized by a T cell hybridoma, which in turn secretes IL-2 upon MHC-II dependent priming (48,64). We therefore hypothesized that, mycobacterial phagosome derived Ag85B could be accessed and cleaved by γ-secretase components. Since a specific chemical inhibitor for γ-secretase (L685,458) is available and has been previously used to block nicastrin activity, we initially tested the effect of L685,458 on antigen presentation by macrophages and DCs infected with the BCG vaccine. Figure 26 shows that L685,458 had a strong inhibitory effect on antigen presentation by both macrophages and DCs. As expected, a positive control of pepstatin, which is an inhibitor of cathepsin proteases, also inhibited antigen presentation. Finally, L685,458 showed a dose- dependent effect on antigen presentation by DCs infected with BCG (Figure 27).

Phagosomes themselves present Ag85B assembled into MHC-II: It has been established that Mtb phagosomes themselves assemble Ag85B into MHC-II and present to BB7 T cells (48,64). We speculated that L685,485 may have non-specific effects on antigen

presentation, including surface expression of MHC-II by macrophages. To rule out such non specific effects, BCG phagosomes were purified from macrophages using sucrose gradients, diluted and incubated directly with BB7 T cells. Figure 28 shows that intact BCG phagosomes treated with L658,485 showed inhibition of antigen presentation from BCG phagosomes compared to those from untreated and naïve macrophages.

siRNA blockade of γ-secretase affects antigen production in macrophages and DCs: Nicastrin and presenilin have been reported to possess protease activity on their own (117-121). To further define whether the three components of γ-secretase are involved in the generation of Ag85B peptide epitope through proteolysis, siRNA knockdown experiments were carried out. APCs were treated or untreated with siRNA against nicastrin, APH and presenilin followed by BCG or Mtb H37Rv infection and antigen presentation. Table-III shows the sequences of siRNA probes, which had a mix of at least two or three different siRNAs, which ensured robust and specific knock-down (Figure 23). Figure 29a (BCG) and Figure 29b (*M.tuberculosis*) show that siRNA knock-down of nicastrin , presenilin and APH components of γ-secretase affected antigen presentation by APCs infected with BCG and to a significant extent with Mtb. Control scrambled siRNA had no significant effect on antigen presentation.

L685,458 treatment of DCs affects subsequent *in vivo* immunogenicity : We have previously demonstrated that DCs infected with BCG vaccine can be used to elicit immune responses in mice following vaccination, which are protective against subsequent challenge with virulent tuberculosis (122). This provided a model to examine the immunogenicity of

DCs infected with BCG. To obtain further evidence that γ-secretase may play a role during *in vivo* immunogenicity, DCs were treated or not with L685458, infected with BCG and injected to mice. Viability of the DCs was >90% as evaluated by LDH release assays. Two weeks later, splenic T cells were assayed for Ag85B specific responses of T cells secreting IFNγ using flow cytometric analysis of T cells harvested from spleens and ELISPOT analysis for Ag85B reactive T cells. Figure 30 illustrates that, DCs infected with BCG elicited markedly better $IFN\gamma^+$ $CD4^+$ T cell response in mouse spleens compared to L685458 treated DCs with BCG. Similarly, ELISPOT analysis of corresponding spleens showed that they contained a higher proportion of Ag85B specific $CD4^+$ T cells (Figure 30). These data strongly suggest that γ-secretase is likely to play a significant role in generating a peptide epitope from phagosome-associated Ag85B, and thus mediate immune recognition of mycobacteria, that are otherwise sequestered within macrophages and DCs.

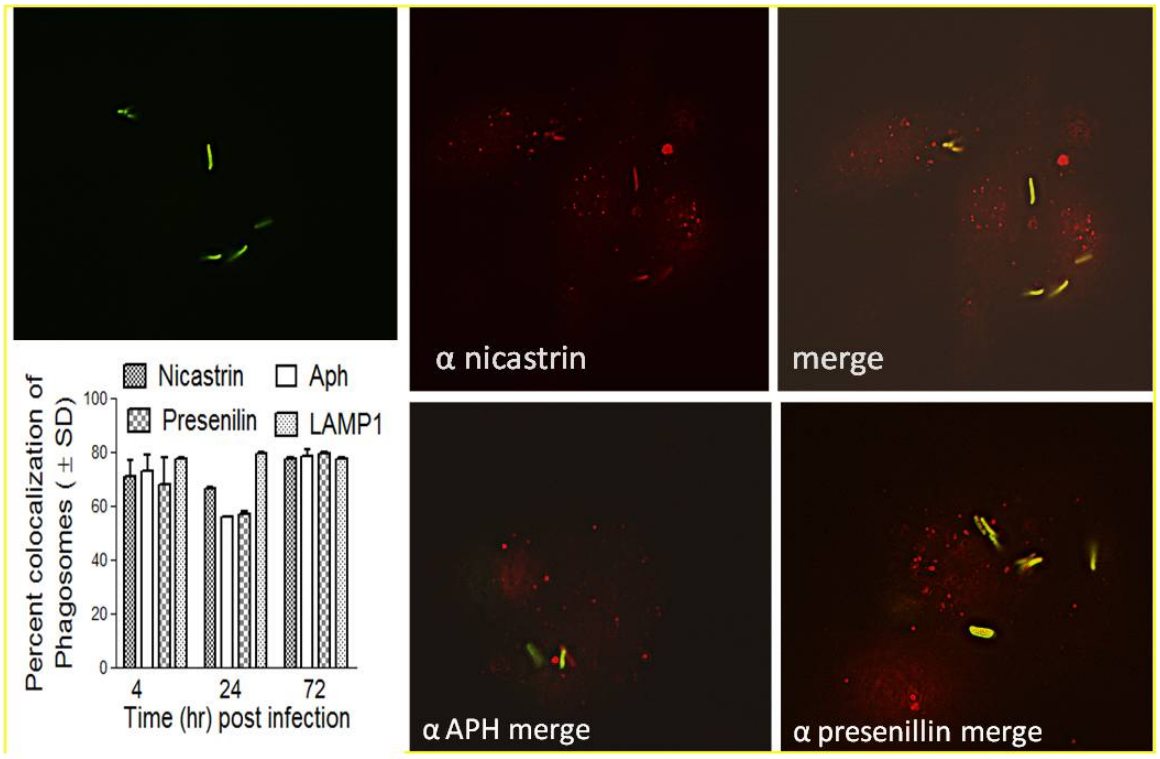


Figure-22: BCG phagosomes within macrophages co-localize with γ-secretase components. *C57BL/6 mice derived primary macrophages were infected with gfpBCG and stained separately with antibodies to nicastrin, APH, presenilin or isotype control followed by Texas-red conjugates. Phagosomes co-localizing with γ-secretase components were scored using laser deconvolution microscopy and plotted. Panels show the three color panels for nicastrin and merged images for APH and presenilin. Isotype did not stain phagosomes and gfpBCG in dendritic cells showed a similar pattern (not shown). Bar graph represents percent co-localizing gfpBCG enumerated in 100 macrophages of triplicate chambers per experiment (n=3 experiments ± SD).*

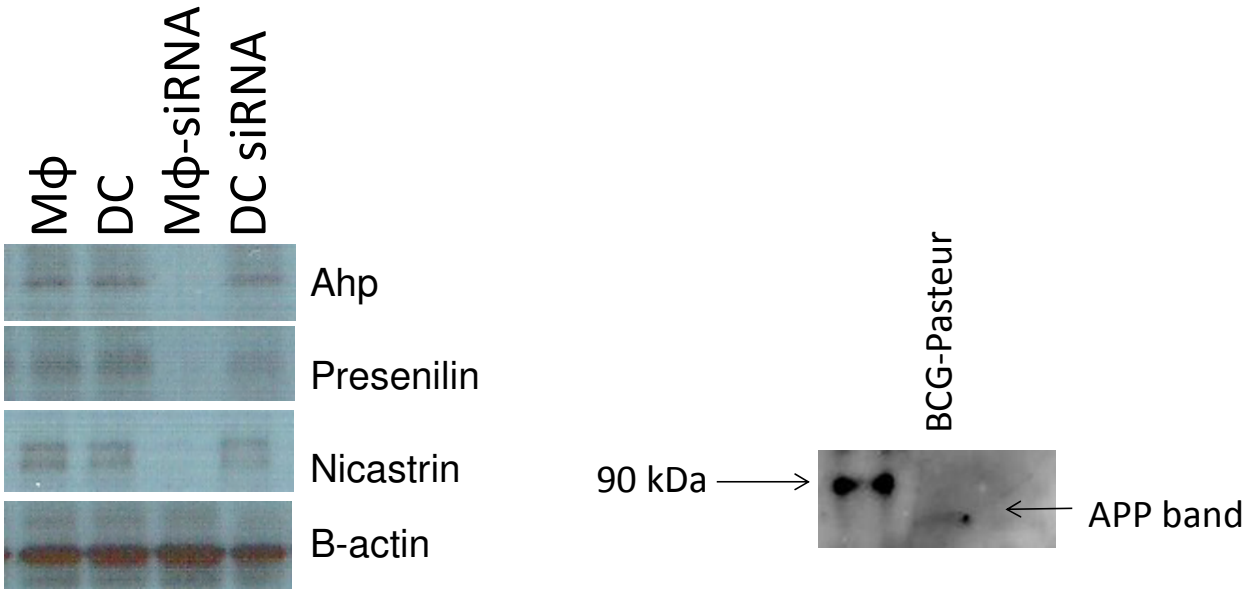


Figure-23 *(left):* ***siRNA mediated down regulation of phagosome associated proteins in macrophages and DCs.*** *Macrophages and DCs were treated with siRNA vs. nicastrin, APH or presenilin and then infected with BCG organisms. After 24 hrs, phagosomes were purified and tested in western blot against antibodies against the proteins. Blot profile shows down-regulation of proteins after siRNA treatment.*

Figure-24 *(right):* ***BCG phagosomes were purified from macrophages on sucrose gradients and analyzed for ability to cleave the amyloid precursor protein (APP).*** *Macrophages were washed and infected with BCG. They were mixed for 4 hours and left overnight. Cells were then scraped and BCG phagosomes isolated using sucrose gradients. Phagosomes were collected and incubated in PBS containing 5 mm EDTA and 0.5 mm 1, 10-phenanthroline for 2 hours. Supernatants were analyzed using western blot and the 87 kDa APP band was detected using an antibody to APP (90 kDa marker).*

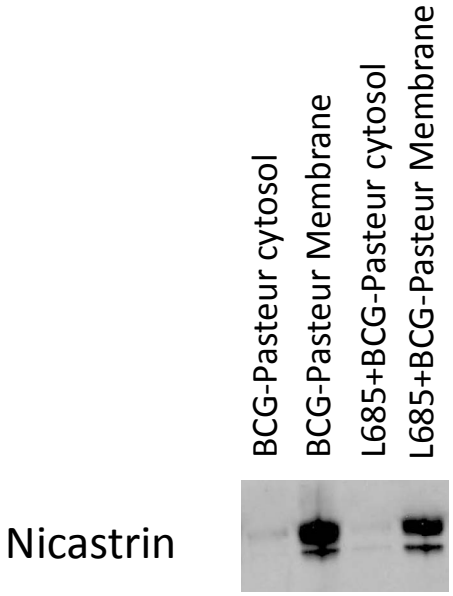


***Figure-25**: **Nicastrin is a membrane associated protease**. Untreated or L685,458 treated macrophages were infected with BCG vaccine strain and 24 hrs later, membrane and cytosol fractions were purified. After protein determination, equal amounts were loaded onto SDS-gels, electroblotted and analyzed for nicastrin using an antibody. Nicastrin is localized only to membrane fractions.*

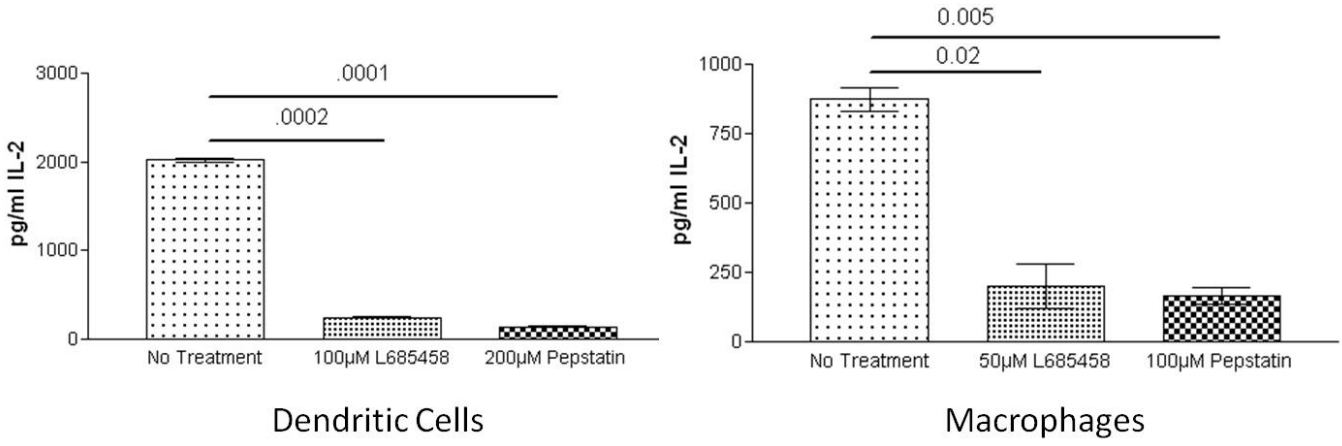


Figure-26: APCs infected with BCG vaccine present mycobacterial Antigen-85B to T cells and the process is inhibited by the γ-secretase inhibitor, L-685458. *The primary APCs from C57BL/6 mouse bone marrow were treated or not with varying doses of γ-secretase inhibitor L-685485, followed by infection with BCG and antigen presentation to BB7 T cell hybridoma. L-685485 and positive control, Cathepsin inhibitor pepstatin, both inhibit antigen presentation.*

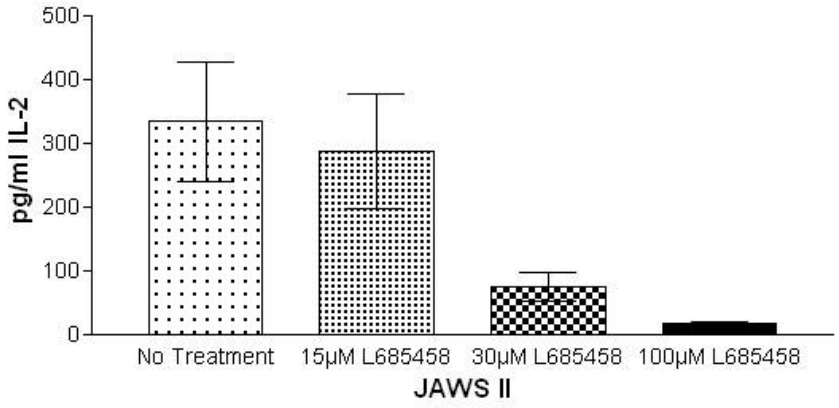


Figure-27: L685458 shows a dose-dependent inhibition of antigen presentation in dendritic cells. *Primary DCs from C57BL/6 mouse bone marrow were treated or not with varying doses of γ-secretase inhibitor L-685485 and Ag85B presentation was evaluated (one of two similar experiments shown).*

Table IIII: Compilation of experiments performed utilizing L685,458

Cell Type	Dose (μM) L685,458	pg/mL IL-2	SD	n
JAWS II	0	334.06	131.26	2
	15	286.81	127.42	2
	30	75.45	32.29	2
	100	16.39	4.16	2
BMA	0	12.61	4.33	2
	15	23.86	16.04	2
	30	25.44	3.67	2
	100	33.16	1.72	2
JAWS II	0	342.88	51.40	2
	15	297.35	188.39	2
	30	56.96	19.85	2
	100	13.34	3.30	2
BMA	0	8.44	2.80	2
	15	8.26	0.60	2
	30	16.52	3.01	2
	100	23.02	0.25	2
JAWS II	0	306.25	44.28	2
	15	218.92	90.54	2
	30	54.30	19.81	2
	100	16.49	1.16	2
BMA	0	5.80	1.32	2
	15	9.83	0.41	2
	30	12.54	2.17	2
	100	19.85	1.22	2
Primary Dendritic Cells	0	492.30	225.69	2
	50	297.01	145.76	2
Primary Macrophage Cells	0	93.08	14.39	2
	50	86.83	1.29	2
Primary Dendritic Cells	0	107.39	10.49	2
	15	65.61	3.68	2
	30	47.64	0.74	2
Primary Dendritic Cells	0	95.03	4.42	2
	15	57.54	9.57	2
	30	47.77	8.28	2
Primary Dendritic Cells	0	155.04	16.75	2
	15	140.33	42.71	2
	30	153.22	101.80	2
Primary Macrophage Cells	0	234.50	12.51	2
	50	205.33	16.58	2
Primary Macrophage Cells	0	873.16	60.66	2
	50	200.16	116.17	2
Primary Macrophage Cells	0	115.37	9.27	2
	50	65.52	0.81	2
Primary Dendritic Cells	0	191.29	3.58	2
	50	145.68	7.17	2
Primary Macrophage Cells	0	3469.13	230.21	2
	50	1485.96	19.71	2

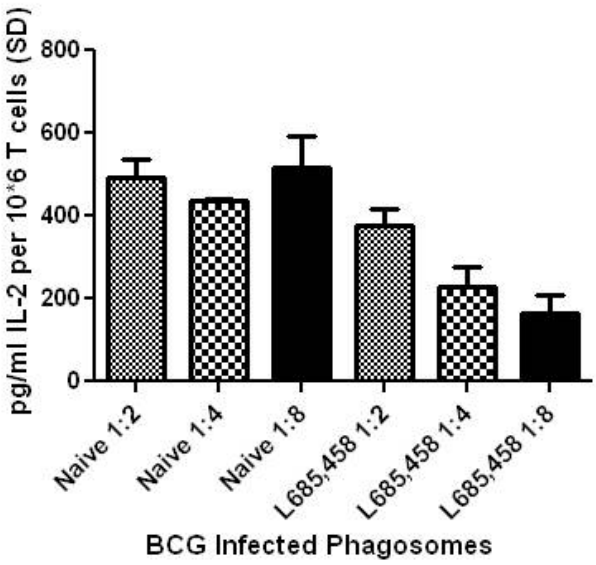


Figure-28: Purified BCG-phagosomes from macrophages present mycobacterial Antigen-85B to T cells and the process is inhibited by the γ-secretase inhibitor, L-685,458. *The primary APCs from C57BL/6 mouse bone marrow were treated or not (naïve) with 100 μM of γ-secretase inhibitor L-685,485, followed by purification of BCG phagosomes which were diluted, and incubated with BB7 T cells (pahosome:T cell ratio). IL-2 released from T cells was measured. L-685,485 inhibits antigen presentation from BCG phagosomes to BB7 T cell hybridoma (one of two similar experiments shown).*

Table-V: *siRNA sequences of probes and controls used to knock-down the components of γ-secretase*

Santa Cruz APH siRNA is a pool of 2 different siRNA duplexes:

• Sense: GGUUUGGCGUUGUCCAUUUtt
• Antisense: AAAUGGACAACGCCAAACCtt

• Sense: CAUCAUUAGUGGUGUAACUtt
• Antisense: AGUUACACCACUAAUGAUGtt

Invitrogen APH siRNA has a sequence of

• Sense: AAAGCGGAACACCUCCUGUAGAAG

Santa Cruz Nicastrin siRNA (m) is a pool of 3 different siRNA duplexes:

• Sense: CGUAGUGGAGAAAGAAGAAtt
• Antisense: UUCUUCUUUCUCCACUACGtt

• Sense: CAACGGCUUGGCUUAUGAAtt
• Antisense: UUCAUAAGCCAAGCCGUUGtt

• Sense: GUGACCCCUUAUCUGACUAtt
• Antisense: UAGUCAGAUAAGGGGUCACtt

Invitrogen Nicastrin siRNA has a sequence of

• Sense: UAUCAUAGACCAUCCGUGAGCUGCC

Santa Cruz Presenilin siRNA (m) is a pool of 3 different siRNA duplexes:

• Sense: GAGUGGAGCUAGAGAUAGAtt
• Antisense: UCUAUCUCUAGCUCCACUCtt

• Sense: CCAGAAUGACAGCCAAGAAtt
• Antisense: UUCUUGGCUGUCAUUCUGGtt

• Sense: CUACGUGUCCAACCAAUCAtt
• Antisense: UGAUUGGUUGGACACGUAGtt

Invitrogen Presenilin siRNA has a sequence of

• Sense: UAGAAACGCACAGACUUGAUAGUGG

The sequence of Control siRNA-A is:

• Sense: UUCUCCGAACGUGUCACGUTT
• Antisense: ACGUGACACGUUCGGAGAATT

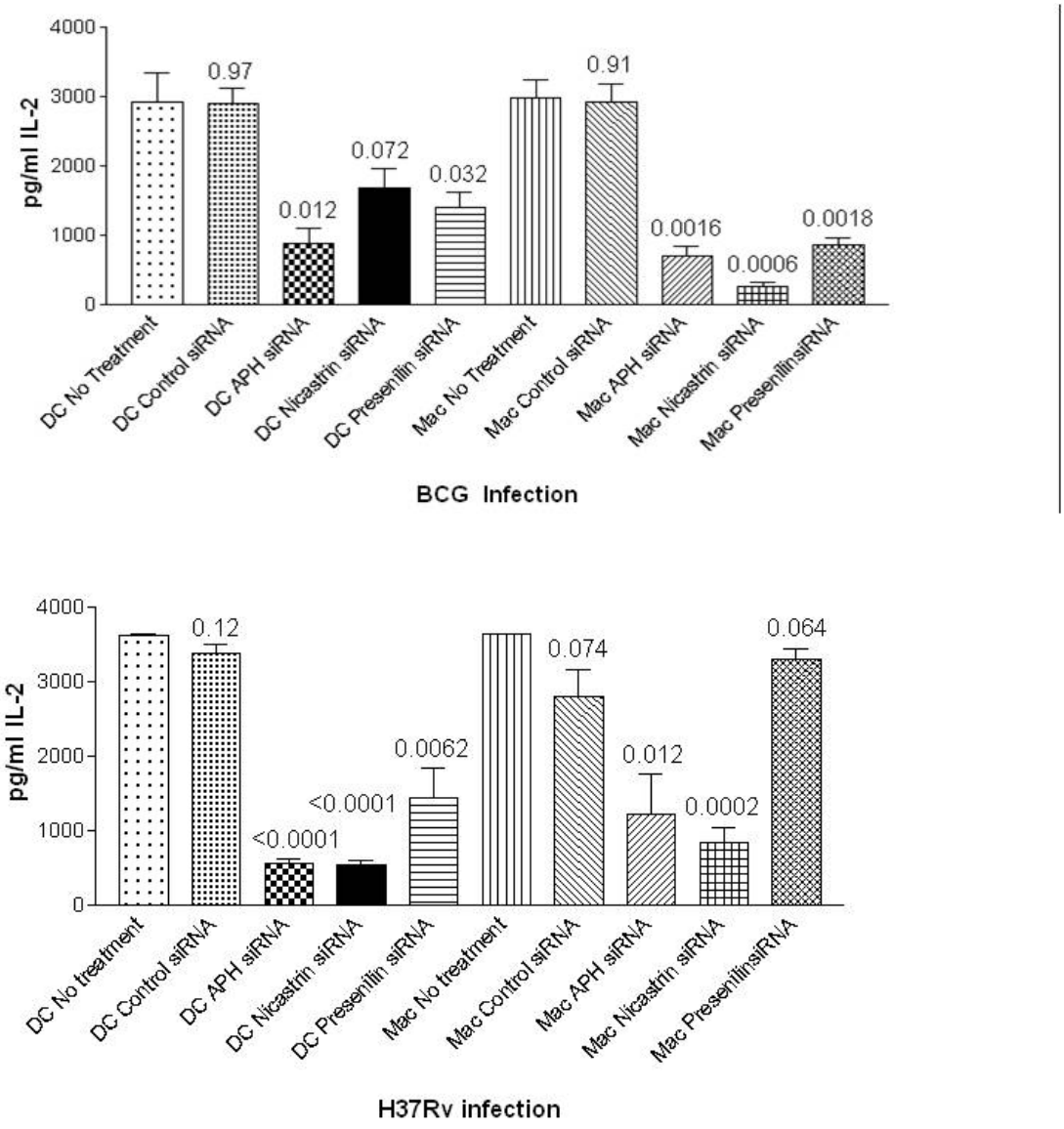


Figure-29: siRNA blockade of the components of γ-secretase leads to inhibition of antigen presentation in APCs. *APCs were treated or not with siRNA against γ-secretase components, followed by infection with either BCG (a: top panel) or Mycobacterium tuberculosis (b: bottom panel) and antigen presentation. siRNA knock down of presenilin and nicastrin significantly affects antigen presentation (p values, student t test; comparison between siRNA control vs. siRNA knock downs).*

Table-VI: Compilation of siRNA knockdown experiments performed

Cell type	siRNA treatment	pg/mL IL-2	SD	N
Primary Macrophage Cells	Naïve	892.41	213.08	4
	Control	916.85	106.86	4
	APH	809.32	434.46	4
	Nicastrin	323.18	100.60	4
	Presenilin	457.76	25.95	4
Primary Macrophage Cells	Naïve	397.90	158.80	4
	Control	692.65	181.09	4
	APH	887.68	30.07	4
	Nicastrin	305.64	152.40	4
	Presenilin	463.24	53.42	4
Primary Dendritic Cells	Naïve	638.96	100.77	4
	Control	1064.84	103.86	4
	APH	802.64	66.00	4
	Nicastrin	1411.81	0.00	4
	Presenilin	151.10	0.00	4
Primary Dendritic Cells	Naïve	1290.17	172.03	4
	Control	1411.81	0.00	4
	APH	677.95	119.83	4
	Nicastrin	1411.81	0.00	4
	Presenilin	806.83	56.72	4
Primary Dendritic Cells	Naïve	2913.92	725.76	4
	Control	2895.27	406.20	4
	APH	883.01	367.51	4
	Nicastrin	1687.40	483.65	4
	Presenilin	1401.41	362.61	4
Primary Macrophage Cells	Naïve	2972.22	461.44	4
	Control	2925.17	449.82	4
	APH	708.91	220.95	4
	Nicastrin	256.00	98.14	4
	Presenilin	859.23	183.03	4
Primary Dendritic Cells	Naïve	3626.89	34.83	4
	Control	3386.24	207.08	4
	APH	552.71	120.99	4
	Nicastrin	536.63	123.17	4
	Presenilin	1433.35	719.01	4
Primary Macrophage Cells	Naïve	3644.30	0.00	4
	Control	2808.92	600.84	4
	APH	1217.64	953.74	4
	Nicastrin	843.05	346.19	4
	Presenilin	3303.93	232.37	4
Primary Dendritic Cells	Naïve	282.42	124.65	4
	Control	168.88	89.35	4
	APH	199.23	96.92	4
	Nicastrin	215.93	85.29	4
	Presenilin	171.82	118.21	4
Primary Macrophage Cells	Naïve	224.61	114.81	4
	Control	178.74	89.03	4
	APH	247.07	137.73	4
	Nicastrin	178.29	31.20	4
	Presenilin	122.92	16.22	4

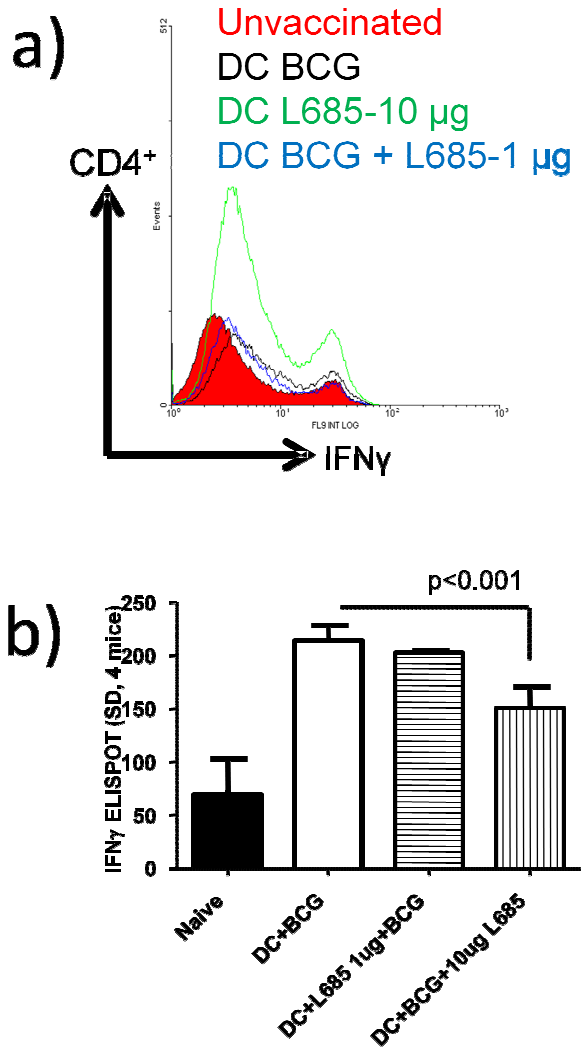


Figure-30: γ-secretase inhibitor L-685458 inhibits the ability of DCs to prime T cells in vivo. *C57BL/6 mouse bone marrow derived DCs were left untreated, treated with a low (1 µg per 10^6 DCs) or high dose (10 µg per 10^6 DCs) of γ-secretase inhibitor L-685,458 followed by infection with BCG. Washed DCs were then injected i.p. to mice. Two weeks later, spleen T cells were analyzed for IFN-γ producing $CD4^+$ T cells (panel a) and Ag85B specific T cells using ELISPOT (panel b).*

CHAPTER 4

DISCUSSION AND CONCLUSIONS

Tuberculosis is a leading cause of death due to infections in mankind. A vaccine is most useful to prevent tuberculosis and BCG vaccine has been used for nearly a century, with partial success. Development of a more successful vaccine is a priority for WHO and various health institutions around the world. In this study, we sought to understand the mechanisms through which antigenic peptides are produced from BCG, which are eventually sorted and presented to the $CD4^+$ T cells through an MHC-II dependent pathway. We initiated our research looking into the immune-evasive mechanisms of *M.tuberculosis,* which is very closely related to BCG vaccine.

The seminal observation by Armstrong and Hart that wild type *M.tuberculosis* sequesters in immature phagosomes of macrophages laid the foundation for the pathogenesis of tuberculosis (123-125). By localizing in immature endosomes and not fusing with lysosomes, wild type Mtb is able to avoid the proteases, lipases and nucleases within the hostile environment of lysosomes. Since lysosomal degradation is the source of peptides that are then sorted into MIIC compartments for MHC-II loading, it seems obvious that the virulent Mtb has an immune evasion strategy. Interestingly, even the attenuated BCG vaccine was reported to be sequestered from human $CD4^+$ T cells, although the molecular mechanisms remained unclear (126). A series of studies following this observation showed that enabling BCG or its antigens to leak into cytosol enhanced its immunogenicity, over-expression of antigens within BCG boosted its efficacy and finally, rapamycin inhibition of the mTOR pathway induces BCG to localize into autophago-lysosomes and increases the vaccine efficacy (122,127-130). These studies have markedly improved BCG vaccine through genetic or immunologic manipulation. However, the mechanism through which the

mammalian immune system recognizes sequestered Mtb pathogen or BCG vaccine within APCs still remains unclear. Such mechanisms are clearly important to devise effective immunization strategies against tuberculosis.

A significant mechanism through which immune recognition can occur could be through processing of antigens secreted from Mtb and BCG. Mtb secretes a number of antigens like Ag85 complex (A, B and C), ESAT-6, CFP-10 and TB10.4 among others. BCG also secretes Ag85 complex and TB10.4, but is deficient in ESAT-6 and CFP-10 (131-134). Since secreted proteins are major immune-dominant antigens in mice and humans, it has been speculated that under natural conditions of infection or vaccination, the immune system mostly recognizes secreted protein antigens through either MHC-I or MHC-II pathways (52,53). This is evident from the overwhelming T cell and antibody responses to secreted antigens of Mtb and BCG vaccine in animal models and humans.

The MHC-II dependent $CD4^+$ T cells appear to be more active against active infection since, HIV-1 infected humans with $CD4^+$ T cell depletion rapidly succumb to tuberculosis. However, MHC-I dependent $CD8^+$ T cells also appear important, although, there seem to be impediments for proteasome-mediated processing of secreted antigens for $CD8^+$ responses. Thus, despite secreting Ag85 complex proteins, BCG is a poor inducer of $CD8^+$ responses and expression of pore-forming listeriolysin toxin was found to enhance $CD8^+$ responses in mice (135). Antigens 85A-B-C and *pstS* are major immunogenic proteins of BCG vaccine, and yet, only a limited number of $CD8^+$ epitopes have been mapped using tetramers in mice. In contrast, most secreted antigens are well known to trigger $CD4^+$ T cells responses,

although, the sorting of secreted antigens, located in the cytosol into MHC-II dependent pathway remains unclear. The processing of secreted antigens of BCG vaccine within phagosomes was therefore a major focus of this study.

We described recently that intact BCG vaccine and its antigens could be routed to lysosome like compartments through induction of autophagy in macrophages and DCs (122). However, Ramachandra et al. described that mycobacterial phagosomes by themselves could assemble peptides *in situ* into MHC-II complexes, which are then exported to plasma membranes for presentation to $CD4^+$ T cells (63,64). In subsequent studies, we used an *in vitro* Ag85B antigen presentation assay to identify that Cathepsin-D was a major protease that cleaved Ag85B to produce a $CD4^+$ T cell epitope (36). Though BCG produced limited amounts of Ag85B epitope to be presented to BB7 T cells, it was interesting because, the pH of BCG phagosomes was near neutral, and the purified BCG phagosomes contained reduced amounts of active 30 kDa Cat-D required to produce Ag85B epitope. Keeping in mind that *in vivo*, phagosome environment could be variable, we asked a question, whether Ag85B epitope production in phagosomes was determined solely by pH dependent activation of Cat-D and similar enzymes or whether other proteolytic processes were present.

Studies described here show that both vATPase and phagocyte oxidase (phox, NADPH oxidase) play a major role in the regulation of lumen pH, but the BCG vaccine derived SOD seems to negate the effects of vATPase and phox by maintaining a neutral pH. Thus, knocking down the expression of SOD through genetic manipulations led to a more immunogenic phenotype for BCG vaccine. This vaccine is currently under clinical trials and

it is a possible candidate to replace BCG wild type vaccine. By investigating the pH regulation processes therefore, we were able to find a novel molecular basis to improve the BCG vaccine. Even after blockade of vATPase and phox, we observed that macrophages still presented antigen to T cells. Thus, we speculated that alternate mechanisms or proteases may be present within the phagosomes.

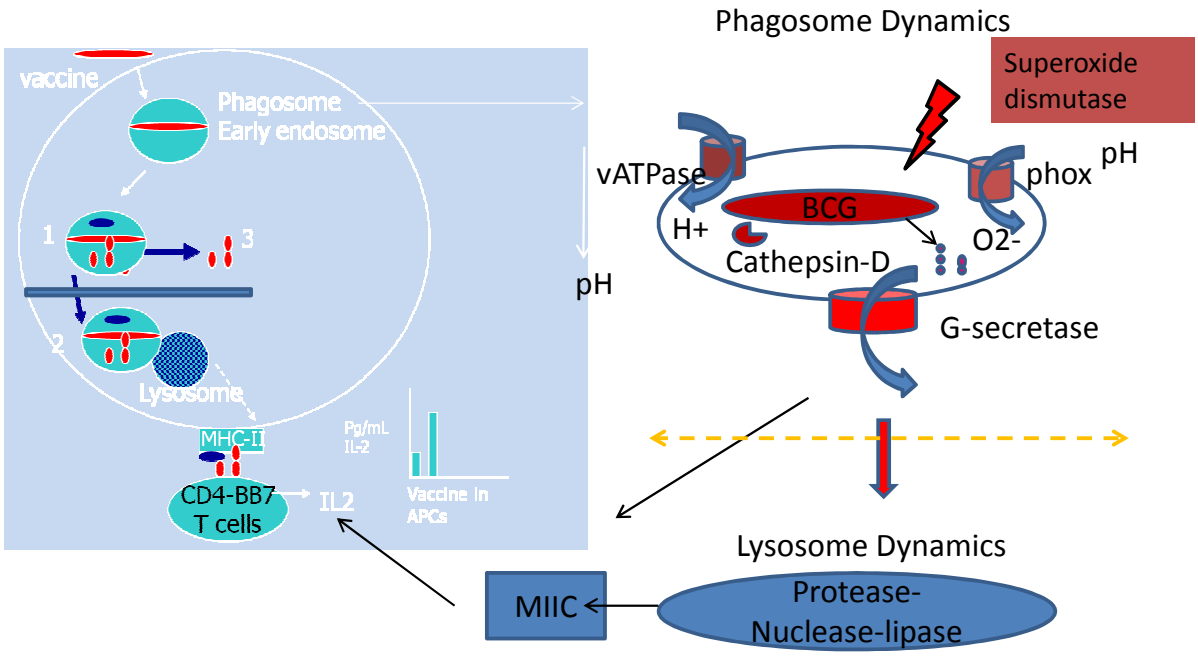
In an attempt to further characterize proteases associated with the phagosome, we then performed a proteomic analysis of BCG and Mtb phagosomes and identified that nicastrin was enriched on phagosomes (92-94). The enrichment of nicastrin on mycobacterial phagosomes was initially identified during a proteomics that was confirmed by others (94,136). Data reported in this study suggest that, nicastrin is associated with a proteolytic cleavage of Ag85B to produce an immune signal. Our observations using proteomic analysis are novel and thought provoking in several directions. First, we had documented earlier that the phagosome pH is near neutral that prevents the generation of sufficient levels of active proteases within the phagosome lumen (36). Being associated with the phagosome membrane, nicastrin may not be affected by the intracellular environment of the phagosome and thus may aid in proteolysis, either at the membrane interface or outside the membrane. We noted that purified BCG phagosomes themselves were capable of priming BB7 T cells in vitro and this was affected by L685458, an inhibitor of γ-secretase. The assembly of nicastrin alone or as a part of γ-secretase on the phagosome membrane may also help to perform a physiological function involved in endosome recycling, as proposed by Jutras et al. (99). Nicastrin or γ-Secretase may therefore fortuitously cleave the mycobacterial proteins helping the immune system to recognize the intraphagosomal mycobacteria. Third,

γ-secretase may potentially produce shorter peptide fragments, and downstream events may channel them towards an alternate $CD8^+$ pathway. We therefore propose that γ-secretase and its components perform an unintended proteolytic function at the phagosome level and this may benefit the host during infections.

It is tempting to hypothesize that we may be able to exploit the proteolysis function of γ-secretase. For example, we can clone and express an immunogenic protein into CD44, a known substrate of γ-secretase and express it within BCG as a secreted protein. Then, one can expect γ-secretase to cleave CD44 as well as associated antigen and produce peptides that could conceivably find their way into antigen presentation. Furthermore, we can use an SOD mutant to express CD44 that will probably facilitate a lumen with acidic environment, and aid proteolysis *in situ*. Future studies can focus on these issues to strengthen the BCG vaccine.

To conclude, a comprehensive examination of events occurring within the BCG vaccine phagosomes has helped to unravel some novel mechanisms that seem to determine the efficacy of the BCG vaccine. Our studies have highlighted that the mycobacterial phagosomes contain in excess of 400 proteins indicating the complexity of the phagosome and the daunting task of a functional characterization. Interestingly, this study illustrates the use of a system biology approach to dissect the function of a phagosome-associated protein. We anticipate that a similar systems-biology approach will enable analysis of other novel proteins associated with the phagosomes and help in understanding pathogenesis and vaccine development.

Figure-31: Summary of findings from this study. Because the BCG vaccine does not fuse with lysosomes, much of the antigen-85B derived epitope is produced within the phagosomes. The phagosomes have two pumps, vTPAse and phagocyte oxidase (phox) that work in opposing directions, reducing pH and increasing pH, respectively. The acidic pH facilitates the generation of 30 kDa Cat-D that can cleave and produce Ag85B epitope, which is then presented to the T cells. The BCG vaccine secretes superoxide dismutase that, in turn, raises pH of the lumen. This leads to reduced levels of antigen production in a neutral phagosome. In addition to, the pH regulating pumps, a third membrane associated protease, γ secretase also seems capable of cleaving Ag85B. Thus, the BCG vaccine phagosome is a critical determinant of immune responses originating from macrophages and DCs.



Chapter 5

Materials and Methods

5-1 PRIMARY MACROPHAGES, DCS, CEL LINES, AND BCG STRAINS

Bone marrow from C57BL/6 mice (M/F)(4-8 weeks) (Jackson research Labs, USA) was obtained by flushing both the femur and tibia. The bone marrow pooled from 2 mice at a time was centrifuged for five minutes at one thousand rotations per minute. The bone marrows were washed two times in ACK lysing buffer (Fisher BW10-548E), followed by one milliliter (ml) ,of phosphate buffered saline (PBS), passed through a 36-gauge needle and suspended in Iscove's modified Dulbecco's medium (IMDM) with ten percent fetal bovine serum (FBS), 20 ng per milliliter granulocyte macrophage colony stimulating factor (GM-CSF), and antibiotics, penicillin and gentamicin. The cells were then plated into six well plates and incubated at 37^{o}C, 5% CO_2 for one week, while spiking the wells with two milliliters of fresh media, every two days. After seven days, the wells are flushed and the cells from several plates are transferred to a 50ml screw cap tube. The cells are pelleted and resuspended in MACS buffer, PBS containing 0.5% FBS, at a volume of 400µl per 10^8 cells. CD11c microbeads (Miltenyi 130-052-001) were then added to the cells, 100µl per 10^8 cells. The cells were incubated at 4^{o}C for fifteen minutes, washed once in 1ml of MACS buffer and resuspended into 500 µl MACS buffer. During this time, the Miltenyi separation columns (Miltenyi 130-042-201) were prepared by rinsing once with 500 µl MACS buffer, one column per 10^8 cells and placed onto magnetic holders. The cells are added to the columns and the flow through is collected. The macrophages will flow through since they do not have CD11c on their surface, thereby not binding the microbeads. The columns are washed three times with 500 µl MACS buffer and then removed from the magnetic holders. One milliliter dissociation buffer is then plunged through the column, thereby washing the dendritic cells off the column. Wash off was repeated once. The flow-through macrophages

were then run through another column repeating the procedure. The cells (DCs and macrophages) are counted and plated into twenty-four well plates with 10^6 cells per well in IMDM supplemented with 10% FBS and GM-CSF and allowed to differentiate for 4-7 days. Medium was replaced with GM-CSF free medium and cells were used for various assays (36). BMA A1, derived from C57Bl/6 mice, macrophage cell line used for obtaining phagosomes and was a kind gift from Dr. Kenneth L. Rock (UMASS, Cambridge, USA).

BCG Pasteur was from ATCC collection, BCG Tice strain and the mutants were donated from Dr. D. Kernodle from Vanderbilt university and gfpBCG was a gift from Dr. Dhandayuthapani, UTHSC-SA.

5-2 ANTIGEN PRESENTATION ASSAY

Macrophages or dendritic cells in 24 well plates were infected with BCG at a multiplicity of infection (MOI) of 5:1. The cells were placed on a shaker at 37^oC, 5%CO_2 for four hours or 18 hrs as per experiment. Monolayers were then washed with fresh medium three times to remove non-phagocytosed bacteria, and overlaid with 250µl of BB7 T cells, at a ratio of 1:20. These cells specifically recognize MHC class II loaded with a peptide from the mycobacterial antigen 85B (aa 241-256: VANNTRLWVYCGNGTP). The plates were either incubated for 4-24 hrs depending upon the experiment. The supernatant was collected and tested for IL-2 using sandwich ELISA kit from Biolegend Inc (USA) (36).

5-3 PROTEIN ASSAY

Protein assays for phagosomes were performed utilizing the BCA Protein Assay Kit (Thermo Scientific Pierce-23225). The protocol followed was as described in the Pierce BCA Protein Assay Kit. Equal amounts of proteins were loaded per lane of SDS gels in all experiments.

5-4 WESTERN BLOTS

Western blot analysis was performed utilizing Bio-Rad pre-cast Criterion gels and gel system. Controls used in all experiments were Pierce Blue Ranger pre-stained markers as well as the Cruz marker (Santa Cruz Biotechnology, CA). SDS gels were run and transferred to Bio-Rad Immuno-Blot Polyvinylidene Fluoride (PVDF) membranes. All primary antibodies were incubated overnight and secondary antibodies for 90 minutes(36). ECL kit was used to develop the blots.

5-5 ENZYME LINKED IMMUNOSORBANT ASSAY (ELISA)

Following the Biolegend protocol, ninety-six well ELISA plates were coated with 100µl per well of IL-2 capture antibody (Biolegend 431003 IL-2 Kit) and incubated overnight at 4^{o}C. The plates were then blocked for two hours with 200 µl PBS containing 1% albumin per well. After blocking the plates, 100 µl of sample, or IL-2 standard was added and the plates were then incubated overnight at 4^{o}C. The ELISA plates were washed with PBS containing 0.05% Tween-20 three times. 100µl detection antibody per well was added to each well and the plates were incubated at room temperature for two hours. The plates were washed again and 100µl of avidin-HRP per well was added and incubated for thirty minutes. The plates were washed for the final time and 100µl Sure Blue TMB 1-component per well peroxidase

substrate (KPL 50-00-01) was added and incubated for fifteen minutes. 50µl Stop solution, 2N H_2SO_4, was added to each well and the plates were read at 450nm. A standard curve was calculated using the IL-2 standards loaded on the plates, and the IL-2 content of samples was determined.

5-6 IMMUNOFLUORESCENT STAINING

Dendritic Cells or macrophages were plated onto 8-chamber glass slides (BD 354118). The slides were incubated overnight at 37°C, 5%CO_2 in activator-free medium or activators, such as Gm-CSF, for 4 or 24 hrs. Cells were infected with *gfp*BCG and placed on a shaker at 37°C, 5%CO_2 for four hours, removed from the shaker and incubated for 4-8 or eighteen hours. Slides were washed with PBS three times and 200µl of 2.7% paraformaldehyde was added to each chamber for fifteen minutes. 200µl of .01mg/ml digitonin in PBS was added to each well and incubated for eight minutes. The paraformaldehyde was aspirated and cells were then incubated in 200µl perm buffer, containing 0.1% saponin and 0.05% tween-20 in PBS, and incubated for thirty minutes. Blocking buffer consisting of 0.1% glycine and 5% heat inactivated mouse serum in PBS was added and slides were incubated for 30 minutes. Primary antibody was added to the slides and they were incubated overnight at 4°C. Slides were then washed thrice with PBS containing 0.05% tween and Texas Red secondary (Jackson Immunoresearch Chemicals, USA) was added to each well and slides were incubated for three hours. Isotype specific antibodies were used in the place of primary antibody as a control in all steps of our experiments. Slides were then washed and rinsed once with cold methanol. Slides were then scoped and analyzed using a Nikon microscope

with Metaview software. Co-localization was confirmed using laser confocal microscopy when needed (36).

5-7 PHAGOSOME FRACTIONATION

The method described by Ullrich et al. to purify mycobacterial phagosomes was used without modifications [135]. Antigen presenting cells (BMA.A1 cell line, CyDC cell line or primary cells) were infected at an MOI of 5:1 with BCG, or other mycobacteria, gently mixed for 4 hours at 37^{o}C, 5% CO_2 and then incubated for eighteen hours. Cells were then scraped and samples were pooled from multiple flasks. Cells were washed three times with PBS and resuspended in an anti-protease mix consisting of 1 µg/mL leupeptin, 1 µg/mL pepstatin and 1 mM phenylmethyl sulfonylfluoride. Cells were then transferred to a glass cell grinder and ground prior to being passed through a syringe with a 27½ gauge needle. Cells were then layered on a gradient composed of 4 mL 50% sucrose and 2 mL of 12% sucrose and centrifuged for 90 minutes at 4^{o}C at 2000 rpm. The interface, composed of phagosomes, was collected after removing the upper 12% sucrose layer (post nuclear supernatant). The phagosomes were pelleted at 12000 rpm for 5 min and the supernatant was discarded. 500 µL of PFB (0.5 mm EDTA, 0.5 mM EGTA, 0.05% gelatin and 20 mM hepes brought to a pH of 7.0) was added and phagosomes were overlaid on 2 ml of 10% Ficoll 70,000 in 5% sucrose, centrifuged at 2000 rpm for 30 min and the interface was collected. This second step was repeated once more to yield > 95% pure phagosomes as judged by electron microscopy (137).

5-8 PROTEOMIC ANALYSIS *(method reproduced from Li et al, IJCLM(92,93))*

Phagosomes were fractionated as noted previously, Methods, prior to further analysis. Samples of 100 µg phagosomes containing BCG, Mtb H37Rv or ΔfbpA were fractionated into five SDS/PAGE gel fractions. Peptide extracts from each fraction were purified before analysis. Analysis was performed with a nanoLC/LTQ-FTMS system at the Proteomics and Informatics Facilities Services in the Research Resources Center at University of Illinois at Chicago (PISF-RRC-UIC), which is supported by the Searle Funds at the Chicago Community Trust. Each extract was injected once and a total of 15 LC/MS injections were performed for the three phagosome samples. With the Sorcerer server (Sage-N Research, Inc.; Milpitas, CA) hosted at the PISF-RRC-UIC, the raw data files were compared to a composite database containing the protein databases of mouse, H37Rv, BCG, and their reversed decoy databases. The raw data files were then converted to mzXML files using the Trans-Proteomic Pipeline software. The mzXML files were searched against the composite database as well. For reference, the peptide mass tolerance was set at 15 ppm with methionine oxidation as a differential modification. Up to 2 missed cleavages and an isotope check using a mass shift of 1.003 amu were allowed with only MS/MS identifications with peptide probability above 0.5 for quantitation being accepted for publication.

5-9 siRNA KNOCKDOWN OF PRIMARY CELLS

A specific approach to inhibit γ secretase component expression is the use of siRNA against APH, nicastrin or presenilin. This procedure from Santa Cruz Biotechnology involves transfecting macrophages or dendritic cells with long double stranded RNA, which upon uptake, enter the RNA interference pathway. The RNA is degraded into 19-21 nucleotide fragments by an RNAase III enzyme called dicer, and subsequently this binds to the

complimentary mRNA sequence. The coupled mRNA is then cleaved prior to translation, thus inhibiting production of the targeted protein. Primary cells are transfected utilizing Santa Cruz's Transfection Reagent and incubated for 30 hours. siRNA-transfected as well as naïve cells were infected with mycobacterial strains for 4 hrs and washed. At this stage, replicate wells of cells were lysed to determine protein content by western blot. Other wells were washed and incubated in medium for 24 h and then overlaid with BB7 T cells. IL-2 was then estimated at several time points of incubation. The controls used are scrambled sequences of siRNA.

5-10 pH DETERMINATION

Macrophages were plated in 8-well coverglass bottom chambers (Lab-Tek, Naperville, IL). After 24 h when the cells were 30–50% confluent, they were incubated in 1 mg/ml of the pH indicator LysoSensor Yellow/Blue dextran (Molecular Probes, Eugene, OR) for 12 h and then washed with cold PBS. Macrophages were acidified using 60 min incubation with nigericin if needed. LysoSensor is an UV-excited fluorophore with emission maxima at 390, 450 and 530 nm that are proportional to pH. This dextran-conjugated indicator is taken up by vesicles that are part of the endocytic pathway and are comprised of endosomes and lysosomes. Higher 530/450 nm ratios correlate with a lower pH. The labeled cells were observed with a fluorescent microscope (Nikon microscope with a Metamorph image analysis software), using excitation at 360 nm and an emission filter at 450 nm and a long pass emission filter at 515 nm. With the dextran-labeled LysoSensor Yellow/Blue dye, the ratio of 530/450 nm emission was calculated for endocytic vesicles by using the MetaMorph Image (or Metaview) processing system (Version 4.5r5, Universal Imaging Corp., PA) and

averaged for each image(138). The 530/450 nm ratio was calculated on a minimum of 100 intracellular phagosomes in each of the four images analyzed per experiment and each experiment was repeated three times (arbitrarily we can score the phagosomes as green and blue and express their ratio to reflect the pH).

5-11 FRACTIONATION OF CYTOSOL AND MEMBRANE FRACTIONS FROM PRIMARY CELLS

Primary macrophages or dendritic cells are plated into 6-well plates at a count of $2x10^6$ cells per well in IMDM with 10% FBS. The APCs are incubated 24 hours prior to treatment or infection. If a treatment is performed on the APCs, they are treated for 24 hours prior to infection, which is then allowed to proceed for an additional 24 hours. The cells were then washed thrice with 1 ml PBS and then incubated on ice with 300 µl digitonin in a sucrose buffer(139,140). The plates are incubated for 10 minutes and the buffer is then collected and pooled. The samples are then concentrated using Centricon centrifugal ultrafiltration vials until a final volume of 100 µl is obtained. This sample is termed the cytosolic fraction. The wells of the plates are then scraped using a cell scraper, and rinsed with 1ml PBS combining wells of the same sample. The samples are then centrifuged at 12000 rpm for five minutes, and washed three times with PBS containing 10% Triton-X and then resuspended in PBS. This portion of the sample is now termed the membrane fraction and stored after protein determination.

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- December 2001-August 2002 — Boston, MA
  Research Assistant II
  Department of Hematology and Oncology, Tufts-New England Medical Center
  Principal Investigator: A. Kuliopulos, M.D., Ph.D.

- August 2000-December 2001 — Cambridge, MA
  Data Manager
  Millennium Pharmaceuticals

- May 1998-August 2000 — Allston, MA
  Laboratory Technician
  Biomedical Laboratories
  Director: Appu Pillai, Ph.D.

Education

- 2005 –Present University of Texas-Houston — Houston, TX
  Ph.D., Molecular Pathology. Expected May 2011
  Title: Novel Mechanisms of Antigen Processing that Enhance BCG Vaccine Efficacy
  Advisor: C. Jagannath, Ph.D.

- 2002 –2005 University of Texas-Houston — Houston, TX
  M.S., Biomedical Science
  Title: Role of Cathepsin-D During Mycobacterial Antigen Presentation
  Advisor: C. Jagannath, Ph.D.

- 1996 –2000 Boston University Boston, MA
  B.A., Biochemistry and Molecular Biology

Memberships

- Student Member: American Society of Microbiology, 2003-Present
- American Association for the Advancement of Science, 2010-2012
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Awards and Presentations

- 2010 Graduate School Education Committee Travel Award
- 2010 American Society of Microbiology Poster Presentation
- 2010 Graduate School of Biomedical Sciences, UT-Houston Student Travel Award Recipient
- 2010 Robert W. and Pearl Wallis Knox Charitable Foundation Scholarship
- 2009 The Changing Landscape of Vaccine Development Symposium Poster Presentation, Galveston National Meeting
- 2009 Texas Tuberculosis Research Symposium Poster Presentation
- 2009 Keystone Symposia Poster Presentation
- 2009 Keystone Symposia NIAID Scholarship
- 2008 Molecular Basis for Infectious Disease Retreat Platform Presenter, UT-Houston
- 2008 Graduate School Education Committee Poster Competition 3rd place, UT-Houston
- 2008 American Society of Microbiology Poster Presentation
- 2007 Research Day Poster Contest 3rd place, UT-Houston
- 2007 American Association of Immunology Poster Presentation
- 2007 National Institute of Health FASEB-MARC Travel Award Recipient
- 2007 Graduate School of Biomedical Sciences Poster Contest 4th place pre-candidacy, UT-Houston
- 2006 The Changing Landscape of Vaccine Development Symposium Poster Award Winner, Galveston National Meeting
- 2006 Annual Graduate Student Award in Science and Engineering for the Rice/Texas Medical Center Chapter of Sigma Xi
- 2006 Academy of Medicine, Engineering and Science of Texas Poster Award for Vaccine Category
- 2006 Bugs, Drugs and Vaccines: Securing Our Future Symposium Tanox, Inc. Travel Award and Platform Presenter, Galveston National Meeting
- 2005 L.D. Mehta Achievement Award
- 2005 McGovern Conference Award Winner Pre-Candidacy, Galveston National Meeting
- 2005 Graduate School of Biomedical Sciences, UT-Houston Student Travel Award Recipient
- 2005 American Society of Microbiology Poster Presentation
- 2005 University of Texas Outstanding Community Service Award

- 2005 McLaughlin Travel Award Recipient, Galveston National Meeting on Bioterrorism
- 2004 Graduate School of Biomedical Sciences, UT-Houston Student Travel Award Recipient
- 2004 American Society of Microbiology Poster Presentation
- 2003 McGovern Conference Platform Presenter, UT-Houston

Extracurricular and Professional Activities

- 2008-2009 Graduate Student Association Vice President
- 2002-Present Graduate School of Biomedical Sciences Student Outreach
- 2005-Present Graduate School Education Committee program representative
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Research Support

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Publications

- **Christopher R. Singh**, Pearl Bakhru, Arshad Khan, Qing Bo Li and Chinnaswamy Jagannath. Nicastrin- a component of γ-secretase generates a peptide epitope facilitating immune recognition of intracellular mycobacteria through MHC-II dependent priming of T cells. *Journal of Immunology:Cutting Edge* (submitted; under revision-2011).

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